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Over the past year, we have continued the optimization of the cell/vector system and the procedures for the selection of genetic suppressor elements (GSEs) conferring drug resistance in MCF7 breast carcinoma cells. We have developed a rapid and efficient procedure for the recovery of integrated retroviruses from the DNA of selected cells. We have also identified retroviral vector LmGCX, which carries green fluorescent protein (GFP) as a selectable marker, as an efficient vehicle for gene expression and GFP selection in MCF7 cells. The LmGCX vector was used to generate a library of normalized random cDNA fragments from MCF7 cells. This library includes 29 x 10^6 clones, >90% of which are recombinant. The cDNA fragments in this library carry different adaptors at their 5' and 3' termini (as oriented relative to the original mRNA), providing translation initiation and termination codons for peptide expression. This library has been used to initiate the selection of GSEs conferring resistance to Adriamycin and methotrexate in MCF7 cells.

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Appendix: four reprints

Introduction

Chemotherapy, based on cytotoxic drugs or hormone antagonists, is widely used as either adjuvant or primary treatment at all stages of breast cancer (1). The drug regimens that are most commonly used for breast cancer include a combination of cyclophosphamide, methotrexate and 5-fluorouracyl (CMF), or doxorubicin (presently the most powerful drug in this group) which is most often used as a single agent in the second line of treatment. In addition, taxol was shown by recent studies to produce significant objective responses in breast cancer. Aside from these chemotherapeutic compounds that are used in different types of cancer, another, breast cancer-specific class of drugs is used in the treatment of estrogen receptor-positive tumors. These agents are antiestrogens, the most widely used of which is tamoxifen (1). The objective of the present grant is to identify genes that determine the sensitivity of human breast carcinoma cells to agents used in breast cancer treatment, including antiestrogens (tamoxifen) and cytotoxic drugs (Adriamycin, cyclophosphamide, methotrexate, 5-fluorouracyl, taxol).

Our approach to the identification of chemotherapeutic sensitivity genes is based on the isolation of genetic suppressor elements (GSEs), derived from such genes and inducing cellular resistance or sensitivity to the corresponding agents. GSEs are short cDNA fragments that counteract the genes from which they are derived by encoding inhibitory peptides or antisense RNAs (2). We have previously developed the methodology for GSE selection from retroviral libraries carrying short random fragments of normalized (uniform-abundance) cDNA from mammalian cells (3-5). Using this approach, we identified several GSEs conferring resistance to anticancer drugs or inducing neoplastic transformation (4,5). The same strategy is being used in the present project to identify GSEs that render breast carcinoma cells resistant to chemotherapeutic agents. GSEs inducing resistance to tamoxifen, as well as Adriamycin, methotrexate and taxol will be cloned by expression selection in MCF7 cells. The cloned GSEs will be used to isolate full-length cDNA sequences of the corresponding genes, and the effects of individual GSEs on hormone responsiveness or drug resistance will be investigated. The genes giving rise to such GSEs would constitute likely determinants of chemotherapeutic sensitivity in breast carcinoma.

Body

1. Status of the project at the beginning of the year

During the previous period of the project, we have developed sublines of MCF7 breast carcinoma cells that were highly susceptible to infection with ecotropic retroviruses. These sublines were subsequently found to have lost their estrogen receptor, making them unsuitable for selection with antiestrogens. We have also analyzed the effects of a GSE derived from the BCL2 apoptosis suppressor gene in MCF7 cells and attempted to select new GSEs conferring

Adriamycin resistance in these cells, using a normalized cDNA fragment library derived from HeLa cells. Because of high experimental variability and negative results observed in these and some other concurrent GSE projects, we set out to characterize the parameters affecting gene expression from retroviral vectors used in GSE selection and analysis. We have identified prolonged selection for a drug-resistance marker and low infection rate as factors that are detrimental to gene expression from such vectors (6). We have also generated the evidence for functional cooperation between different GSEs (7), indicating the need for the selection of multiply infected cells and for complete recovery of integrated GSEs at intermediate rounds of selection. Based on this analysis, we developed a series of retroviral vectors that contained Green Fluorescent Protein (GFP) (8-10) instead of a drug-resistance gene as a selectable marker. GFP selection allows one to avoid the detrimental effect of continuous drug selection on the expression of the unselected gene from the same vector, and it also permits the isolation of multiply infected cells on the basis of their higher fluorescence. Fluorescence-activated cell sorter (FACS) selection of GFP-expressing cells was shown to be suitable for the isolation of infectants in MCF7 cells. For improved GSE testing and for the selection of GSEs with potential growth-inhibitory effects, we developed β-galactoside-regulated inducible retroviral vectors (11). To enable regulated expression from these vectors, we have transfected several tumor cell lines, including MCF7, with the bacterial LacI repressor gene, modified for nuclear localization in mammalian cells. We have also generated a normalized cDNA fragment population from MCF7 cells.

2. Development of an efficient procedure for recovery of integrated retroviral vectors.

The finding that GSE effects may be due to cooperation between two or more different GSEs (7) indicated the need to maximize the efficiency of the GSE recovery at intermediate stages of selection. For this purpose, we developed an efficient procedure for rapid rescue of integrated proviruses. In this technique (12), we use "long and accurate" PCR with primers corresponding to the ends of Moloney virus LTR to amplify the entire integrated provirus from genomic DNA. The PCR product is then used directly to transfect BOSC 23 ecotropic retrovirus packaging cells (13), yielding retrovirus as efficiently as the same amount of retroviral vector in the plasmid form. Although we had some initial difficulties using this technique with suboptimal quality DNA preparations or with murine genomic DNA, we found that the provirus could be efficiently rescued from such preparations by doing an additional round of PCR on size-separated products of the first round. The retrovirus population rescued by this "long-vector PCR" (LV-PCR) technique showed excellent conservation of sequence representation (12). The development of the LV-PCR rescue technique has made it much more practical to carry out multiple rounds of GSE selection.

3. Identification of the GFP vector for GSE selection in MCF7 cells and development of estrogen-receptor positive MCF7 cells infectable with ecotropic retroviruses.

In the previous year, we have generated a series of retroviral vectors expressing different forms of GFP and demonstrated the feasibility of FACS selection for MCF7 cells infected with such vectors. In the current year, we tested four of these vectors, carrying the luciferase reporter gene, for the efficacy of luciferase expression in the infected (unselected) populations of MCF7 cells. The highest luciferase expression was obtained with a vector termed LmGCX; expression from LmGCX in MCF7 cells was similar to that from the most efficient neo-containing LNCX vector (14) (after correction for differences in the infection rates). LmGCX contains a "humanized" version of a "red-shifted" (S65T) mutant form of GFP (12), which is expressed from a modified form of Moloney LTR containing a mutation in the U3 region; this mutation ensures efficient expression from an LTR promoter in a broader variety of cell types (15). The gene or GSE inserted in this vector is expressed from a strong cytomegalovirus promoter. The LmGCX vector was chosen for GSE selection in MCF7 cells.

The previously generated sublines of MCF7 cells transfected with the ecotropic retrovirus receptor lost most of their estrogen receptor (ER) and therefore were unsuitable for tamoxifen selection, though still suitable for selection with cytotoxic drugs. To generate an ER-positive cell line susceptible to ecotropic retroviruses, we have transfected an ER-positive MCF7 line (obtained from ATCC) with a plasmid carrying the ecotropic virus receptor gene (16) in pBabeBleo vector containing the phleomycin resistance marker (17). Three independent transfections were done; from each experiment, about 35 highly phleomycin resistant clones were isolated. About 90 clones were tested so far by infection with GFP vectors. Only one clone (termed e/26) showed sizable (22%) infection rate, while the others had only 2-5% infection (in contrast, the previously derived ER-negative MCF7 line showed 60% infection). The e/26 clone will be selected again with phleomycin and further testing will be done prior to using these cells for tamoxifen selection.

4. Generation of a normalized cDNA fragment library from MCF-7 cells in a GFP vector

We have previously generated a normalized cDNA fragment population from MCF7 cells. The cDNA fragments were prepared by a new procedure which allows us to determine the orientation of the cloned fragments relative to the original mRNA. Instead of using random hexanucleotides as primers for reverse transcription, we have used a primer with a defined sequence, containing translation termination codons in all three reading frames (in antisense orientation) as well as a restriction site for cloning, coupled with six random nucleotides at the 3' end of the primer. After reverse transcription with this primer and second-strand cDNA synthesis, double-stranded cDNA fragments were ligated to an adaptor containing three translation initiation codons; this adaptor

was subsequently removed from the 3' end of the cDNA fragments by restriction enzyme digestion. These asymmetrically tagged cDNA fragments were amplified by PCR and used for subsequent normalization, carried out by the procedure of Patanjali et al. (17). The normalization in this cDNA population had been demonstrated by similar signal intensities in Southern hybridization with α-tubulin, 28S ribosomal RNA, c-fos and *MDR*1 probes. We have now cloned this normalized cDNA population into the retroviral vector LmGCX. The resulting MCF7 library contains 29 x 10^6 clones, >90% of which are recombinant; six randomly picked clones were sequenced and shown to contain the appropriate adaptors at both ends; the position of these adaptors allows us to determine fragment orientation relative to the original mRNA.

5. First-round selections for GSEs conferring resistance to Adriamycin and methotrexate

We have used the above described MCF7 retroviral library to infect an ecotropic-virus susceptible subline of MCF7 cells (with low ER levels) and to initiate the selection of GSEs conferring resistance to Adriamycin (ADR) and methotrexate (MTX). For each of these selections, 1.15 x 10^8 cells were used for infection with the library (virus was generated by transfecting BOSC 23 packaging cells); 16% of the cells (18.4 x 10^7 total) were infected. ADR selection was carried out using 120 nM ADR for 24 hours and surviving cells were harvested two weeks later (in preliminary experiments, approximately 0.3% of the cells survived this selection). MTX selection was carried out using 10 μM of MTX for four days; cells were allowed to grow for about two weeks before harvesting (cell survival under these conditions is <0.5%). FACS analysis of GFP fluorescence did not show any changes on the percentage of infected cells after either ADR or MTX selection, which was to be expected at this first round, given the relatively high background of surviving cells and the anticipated low frequency of GSEs (about 1 in 10^6, based on prior experience). DNA was extracted from the surviving cells, and the integrated proviruses are presently being recovered by LV-PCR. The recovered proviruses will be used for the second round of selection.

Conclusions

We have carried out the optimization of the vectors and procedures for the isolation of GSEs conferring drug resistance in MCF7 breast carcinoma cells. Following the analysis of parameters determining GSE expression and functional activity in retroviral vectors, we have developed new vectors, GSE isolation procedures and recipient cell lines. We have also constructed a normalized random fragment cDNA library from MCF7 cells in a vector optimized for expression and selection in these cells and used this library to initiate the selection of GSEs conferring Adriamycin and methotrexate resistance. While these drug selections are conducted in a recipient cell line which has low levels of estrogen receptor, we are currently developing an estrogen receptor-positive

recipient cell line susceptible to infection with ecotropic retrovirus, which will be used for the selection of GSEs conferring resistance to tamoxifen.

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Effects of Infection Rate and Selection Pressure on Gene Expression from an Internal Promoter of a Double Gene Retroviral Vector

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Abstract—*Many commonly used retroviral vectors express one gene from the viral long terminal repeat (LTR) promoter and another gene from an internal promoter. We have investigated factors affecting the expression of the luciferase reporter gene from the internal cytomegalovirus-derived promoter of the retroviral vector, LNCX, which contains a LTR-driven* neo *gene as a selectable marker. A subline of human HT1080 cells, expressing the murine ecotropic receptor, was infected with retrovirus generated by transient transfection of BOSC 23 packaging cells. Mass populations of cells infected under conditions resulting in different initial infection rates (IIR) and selected with G418, showed highly variable luciferase activity. Luciferase expression in cell populations with IIR* $\leq$ *5% was generally low; many populations with IIR* $<$ *1% had marginal or no luciferase activity. The loss of luciferase expression in low-IIR populations was associated with G418 selection. In contrast, cell populations with IIR* $\geq$ *6% showed higher luciferase expression, which was strongly correlated with the IIR. Southern hybridization analysis showed that most cells of the low-IIR populations carried one integrated provirus, with a high incidence of structural rearrangements that abolished luciferase activity. In contrast, populations with IIR* $\geq$ *6% contained two or more copies of integrated provirus per cell, and their luciferase activity correlated with the provirus copy number. Luciferase expression was relatively stable in the populations with IIR* $>$ *1% maintained in the absence of G418. Increasing the selective concentration of G418 or prolonged maintenance of cell populations in the presence of G418 resulted in higher incidence of provirus rearrangements and decreased luciferase expression. These results indicate that the negative effect of selection for the LTR-driven gene on gene expression from an internal promoter depends on the selection stringency and can be obviated by increasing the infection rate.*

INTRODUCTION

Retroviral transduction provides one of the most efficient means for stable gene transfer in a wide variety of mammalian cell types, both *in vitro* and *in vivo*. It is also the easiest of all the virus-based transduction techniques, which was recently simplified by the development of improved transient transfection procedures for generating high titers of recombinant retroviruses (1, 2). The high efficiency of retroviral transduction allows one to analyze phenotypic effects of transduced genes in mass populations rather than individual clones of infected cells, thus avoiding the problems associated with clonal variability. In many experimental situa-

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tions, however, the efficacy of retroviral infection is limited by the nature of the recipient cells (e.g., cells that express low levels of retroviral receptor or cells growing in suspension may be poorly infectable) or by experimental variability in the transfection of packaging cells or tissue culture conditions. Since it is not always feasible to infect 100% of the recipient cells, population assays usually utilize double-gene vectors that express a dominant selectable marker in addition to the gene of interest. Such markers allow one to select a population consisting entirely of infected cells, regardless of the infection rate. The most commonly used retroviral vectors express one gene from the viral long terminal repeat (LTR) promoter and the second gene from an internal promoter (in either sense or antisense orientation relative to LTR), though single-promoter bicistronic vectors containing an internal ribosome entry site (IRES) have gained popularity in recent years (3, 4). The use of an internal promoter, rather than LTR, becomes particularly important when such a promoter provides cell-type specific or inducible expression.

It has long been known that the two promoters of a double-gene retroviral vector are not always coexpressed, and that functional selection for one promoter can lead to inactivation of the other (5). Another well known problem, which is not limited to double-gene vectors, is the instability of gene expression from integrated proviruses (6, 7). A number of studies have addressed the parameters that affect the level or the stability of expression from retroviral vectors (7–16). Several important factors have been identified, including the nature of the recipient cells, relative strength and orientation of the promoters, virus titer, provirus copy number and integration sites, and maintenance of selective pressure. The role for some of these factors, however, remains controversial, with different correlations reported for different cell/vector systems. For example, maintaining the selection pressure was reported in some studies to increase (7) and in other studies to decrease (12) the stability of expression of the nonselected gene. In several studies, the presence of more than one copy of integrated provirus per cell was correlated with higher expression levels and stability (11, 12). Multicopy integration in infected cell populations has been achieved through multiple rounds of infection or by using very high virus titers (12, 14, 17).

Our laboratory has extensively utilized double-gene retroviral vectors carrying the *neo* (G418 resistance) gene to analyze phenotypic effects of different genetic elements in mass populations of infected cells (17–19). In some cases, however, we encountered difficulties in reproducing the same phenotypic effect in different independently derived populations. Experimental variability was primarily observed in the experiments where the infection rates were low and G418 selection was carried out prior to phenotypic analysis. In the present study, we have investigated the effect of differences in the infection rate or in the conditions of G418 selection on the expression of a reporter gene from an internal cytomegalovirus (CMV)-derived promoter of a commonly used double-gene retroviral vector, LNCX (20).

MATERIALS AND METHODS

Vectors and Cell Lines. The Moloney virus-based retroviral vector LNCX (20) was a gift of A.D. Miller (Fred Hutchison Cancer Center). LNCluc was constructed by B.-d. Chang in our laboratory by blunt end ligation of the 1.7 kb firefly luciferase gene, excised with StuI from pGEMluc vector (Promega), into the unique HpaI site located downstream of the CMV promoter in the LNCX vector. BOSC 23 ecotropic packaging cell line, a derivative of human 293 cells (1), and their amphotropic counterpart, BING (21), were a gift of W.S. Pear and D. Baltimore (MIT). PE501 and PA317, murine ecotropic and amphotropic packaging cells (20, 22), were a gift of A.D. Miller. HT1080 pJet-2fTGH cell line (kindly provided by G.R. Stark, Cleveland Clinic Research Foundation, Cleveland, Ohio) was generated by

cotransfection of HT1080 2fTGH line (23) with pJET plasmid expressing the murine ecotropic receptor (24) and a plasmid expressing the puromycin-resistance gene. The original HT1080 cells (without ecotropic receptor) and NIH 3T3 cells were obtained from ATCC. All cells were grown at 37°C in a 7% CO_2 atmosphere, in DMEM supplemented with 10% fetal calf serum and with penicillin-streptomycin.

Retroviral Infection and Isolation of Infected Cell Populations and Clones. BOSC 23 or BING packaging cells were transfected with retroviral plasmid vector essentially as described by Pear et al. (1), except that 2 × HeBS buffer (25) was used instead of 2 × HBS. Murine packaging cell lines PE501 or PA317 were transfected by a standard calcium-phosphate precipitation protocol (25). Packaging cells were plated 24 h before transfection at a density of 2×10^6 or 0.2×10^6 per P60 plate for the human or mouse cells, respectively. The variations in the virus titers were achieved by using different amounts of vector DNA mixed with salmon sperm genomic DNA (carrier) up to the total DNA amount of 15 μg (higher amounts of vector DNA were used in some experiments, as indicated). Virus-containing media supernatants were collected 1 and 2 days after transfection.

In some experiments, the virus titers were determined by a colony-forming assay on NIH 3T3 cells. In this assay, 10^4 NIH 3T3 cells were plated in each well of a six-well plate. 24 h later, cells were infected with 2 ml of virus-containing media, prepared by serial dilutions of the corresponding supernatants from the transfected packaging cells. Cells were incubated with the virus in the presence of 4 μg/ml polybrene (Sigma) for 24 h, then with fresh media for another 24 h, and then with media containing 0.6 mg/ml G418 (active drug concentration) until the cell death was complete in control plates containing uninfected cells. G418-resistant colonies were stained with crystal violet and counted.

1×10^5 recipient HT1080 cells were plated per P60 24 h before infection. For infection, media on the plates were replaced on two consecutive days (unless indicated otherwise) with filtered virus-containing supernatants supplemented with 4 μg/ml polybrene. 24 h later, media were changed and the cells were allowed to grow in fresh media for 48 h more before splitting at 0.5×10^6 cells per P100 (up to 2×10^6 cells per P100 were plated after infections with very low infection rate) and plating in the presence of 0.4 mg/ml (or other indicated effective concentrations) of G418. In each experiment, a control G418-containing plate was seeded with the same number of uninfected cells. G418-containing media were replaced every 3 days; the end of G418 selection was indicated by the time of complete death of uninfected cells. Unless indicated otherwise, the infected populations were maintained in the absence of G418 after the completion of the initial selection. In parallel with the selection of mass populations, the infection rate was determined by colony survival assays. For these assays, 500 cells were plated per P100 without G418 and several different cell numbers (from 10^3 to 2×10^6 cells per P100, depending on the anticipated infection rate) were plated in the presence of G418. At the end of G418 selection, colonies were stained with crystal violet and counted. The infection rate was defined as the ratio of the percentages of cells giving rise to colonies in the presence and in the absence of G418.

Individual cell clones were derived in some experiments from G418-selected populations, by plating such populations at low density (50 cells per P100), in the absence of G418 and picking well-isolated colonies. In other experiments, subclones were isolated during the initial G418 selection, in parallel with the selection of mass populations, by picking individual colonies from G418-containing plates seeded at a lower density.

Luciferase Assays. For luciferase assays, 0.5×10^6 aliquots of cells from different G418-selected populations or clones were collected in exponential phase of growth, suspended in 0.5 ml lysis buffer (Luciferase Assay

System, Promega) and cleared lysates were frozen at −70°C until the assay. Luciferase activity was evaluated using the Luciferase Assay System (Promega) according to the manufacturer's protocol, except that 5 µl of each sample and 50 µl of assay buffer were used for each assay. The luciferase activity was measured in a Beckman LS 5000TD scintillation counter. The results were normalized for the total protein amount in each sample, measured by a modified Bradford method using a Protein Assay Reagent (Bio-Rad). This assay was highly reproducible, as aliquoted frozen cell lysates, repeatedly measured at different times (up to 3 times over 6 months), showed ≤10% variability.

Southern Hybridization. Genomic DNA was prepared using the Qiagen Blood and Cell Culture DNA kit (Qiagen). *Eco*RI or *Hind*III digested DNA was separated by electrophoresis in 0.8% agarose gel and transferred onto HybondN (Amersham) nylon membrane. Hybridization probes were prepared by restriction enzyme digestion of the LNCluc plasmid, followed by the isolation of the desired fragments by electrophoresis in 1% agarose gel and purification with a Qiaquick Gel Extraction kit (Qiagen). Probes were labeled with ^{32}P by random priming, using the Multiprime DNA Labeling System (Amersham). Hybridization and high-stringency washing were carried out as previously described (26).

Copy number of integrated proviruses was determined from Southern blots of *Eco*RI-digested DNA, by measuring hybridization signal intensities using Betascan (Betagen). The copy number for a given band was determined from its signal intensity relative to the signal intensity of the lanes with DNA from a cell line containing a single provirus (clone 1 or clone 2 derived from 0.2%-IIR population). As a control for variability of loading and transfer, the probe bound to the filters was allowed to decay for three months and the filters were then rehybridized with an unrelated probe pMDR2 (27). Relative signal intensities for each band hybridizing with the retroviral probe were normalized for the intensity of the corresponding 3.1 kb pMDR2-specific band (28).

RESULTS

Relationship Between the Infection Rate and the Amount of Vector DNA Transfected into BOSC 23 Packaging Cells. Replication-deficient ecotropic retrovirus LNCluc (Fig. 1), carrying the firefly luciferase (*luc*) reporter gene under the control of the internal CMV promoter of the LNCX vector (20), was prepared in most experiments by transient transfection of the corresponding plasmid vector into ecotropic packaging cells BOSC 23, a derivative of 293 human renal cell line (1). As the recipient cell

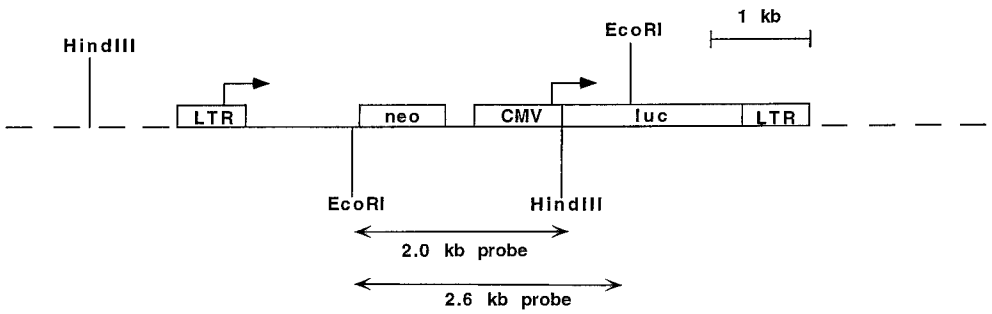


Fig. 1. Map of integrated LNCluc provirus and probes used for Southern hybridization analysis. The arrows show the positions of transcription initiation for the two promoters. Dashed lines indicate the flanking cellular sequences. The 2.0 kb probe was used for hybridization with *Hind*III digests of genomic DNA, where the size of its hybridizing fragments depends on the position of a *Hind*III site in the upstream cellular flanking sequences (placed at an arbitrary location in the figure). The 2.6 kb probe was used for hybridization with *Eco*RI-digested DNA, where, in the absence of proviral rearrangement, it hybridizes to a band of the same size.

line, we have utilized subline pJet-2fTGH of human HT1080 fibrosarcoma cells (a gift of G.R. Stark), susceptible to ecotropic retrovirus and derived by transfecting 2fTGH subline of HT1080 (23) with the murine ecotropic receptor (24).

We were interested in determining the optimal amount of vector DNA for BOSC 23 transfection. For this purpose, recipient cells were infected on two consecutive days with virus-containing media supernatants collected 1 day and 2 days after transfection of BOSC 23 with different amounts of plasmid vector DNA. Infection rates were determined as the percentage of HT1080 cells surviving G418 selection, carried out at the minimal dose of drug that was required for complete killing of uninfected cells (0.4 mg/ml). Figure 2 shows the relationship between the amount of transfected DNA and HT1080 infection rates in six experiments, carried out at different times using as vectors LNCX, LNCluc or LNCX carrying a short (188 bp) cDNA fragment of a mammalian gene. The infection rates reached a plateau at the amounts of transfected DNA that ranged from 0.1 to 5 μg per plate in different experiments. The infection rates at the plateau ranged from 31% to 78%. The experiment yielding the lowest infection rates was conducted with BOSC 23 cells that had been exposed to room temperature for a prolonged period of time; unless strictly maintained at 37°C, BOSC 23 is prone to lose the transgenes that express retroviral proteins (W.S. Pear, personal communication).

In several experiments, the virus yield was estimated on mouse NIH 3T3 cells by the formation of G418-resistant colonies (NIH 3T3 CFU assay). BOSC 23 cells, when rigorously kept at 37°C, consistently yielded a titer of 2×10^6 to 5×10^6 CFU/ml, even when the amount of transfected DNA was as low as 100 ng (data not shown). In three HT1080 cell infection experiments, where the apparent multiplicity of infection (MOI) values (calculated from the NIH 3T3 CFU assays) were 30, 45 and 75, the respective HT1080 infection rates were 71%, 73%, and 41%. On the other hand, infection at a low MOI (0.15) yielded HT1080 infection rate of 3%. Because of a lack of direct correlation between the MOI and the infection rates (as also noted in ref. 29) only the HT1080 infection rates were determined for all of the experiments. These initial infection rates (IIR) were used as a characteristic of the G418-selected populations obtained from the corresponding infections. After the completion of the initial selection, the infected populations were maintained in the absence of G418 (except where indicated otherwise).

Relationship Between IIR and Reporter Gene Expression in G418-Selected Populations. We have measured luciferase activity in 36 populations of HT1080 pJet-2fTGH cells, that were independently infected with LNCluc at different

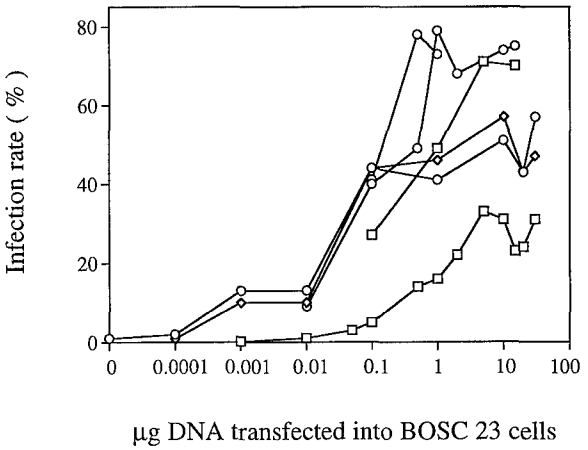


Fig. 2. Relationship between the infection rate and the amount of vector DNA transfected into BOSC 23 packaging cells. The vectors used were LNCluc (squares), LNCX (circles), and LNCX carrying a short cDNA insert (diamonds). The infection rates were determined as the percentage of HT1080 cells surviving G418 selection.

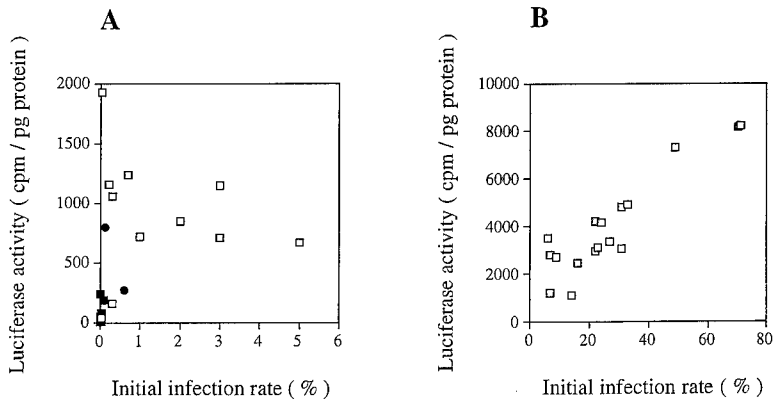


Fig. 3. Relationship between IIR and luciferase activity in 36 mass populations of HT1080 pJet-2fTGH cells independently infected with LNCluc, with IIR $\leq$ 5% (A) or IIR $\geq$ 6% (B). Populations obtained after infection utilizing BOSC 23 packaging cells are indicated with open squares, and populations derived through PE501 or PA317 packaging lines are indicated with closed squares or closed circles, respectively. Luciferase activity was measured on cells recovered in an exponential phase of growth 3–6 days after the completion of G418 selection.

IIR and selected with G418 (Fig. 3A,B). Even though G418 selection assured that each population consisted entirely of infected cells (as indicated by complete killing of uninfected cells in parallel plates), the luciferase activity varied widely among these populations. 9 populations expressed luciferase at very low levels (<300 cpm/pg protein) or not at all; all of these populations had IIR < 1% (Fig. 3A). 9 of the other 10 populations with IIR $\leq$ 5% showed luciferase activities ranging from 600 to 1200 cpm/pg, and one population had 1900 cpm/pg. There was no significant correlation between IIR and luciferase levels among the 19 populations with IIR $\leq$ 5% (r^2 = 0.060) (Fig. 3A). In contrast, 15 of 17 populations with IIR $\geq$ 6% expressed >2000 cpm/pg of luciferase, and the luciferase activity in these 17 populations was strongly correlated with IIR (r^2 = 0.837) (Fig. 3B).

Clonal variability in luciferase expression was analyzed in cell lines derived from infection experiments with high or low IIR values. Fig. 4A shows the luciferase levels in cell lines that

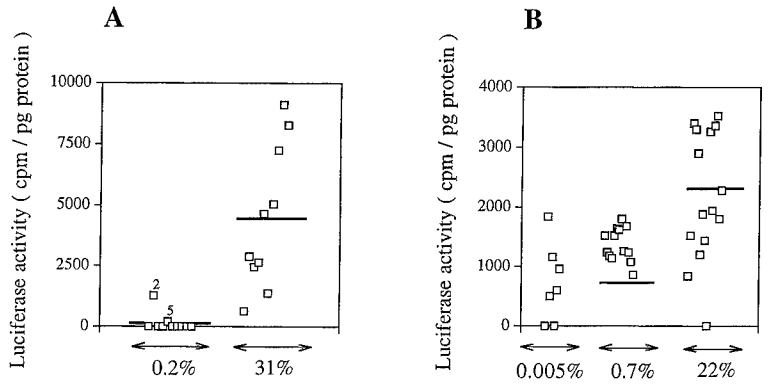


Fig. 4. Luciferase activity in clonal cell lines derived from different mass populations of infected cells. Each set is designated by the IIR values (%) of the corresponding populations. Squares indicate the luciferase activity of individual clones, and horizontal lines show the activity of the corresponding populations. Clones in (A) were derived by subcloning from the corresponding G418-selected populations; clones in (B) were isolated as individual colonies in parallel with the corresponding mass populations. Clones 2 and 5 from the 0.2% IIR population are labeled in panel A.

were derived by subcloning (in the absence of G418) from G418-selected populations with a low (0.2%) and a high (31%) IIR. Clones derived from the high-IIR population showed highly variable luciferase expression levels, with the average level similar to the luciferase activity measured in the parental population. In contrast, 8 of 10 clones derived from the low-IIR population showed no luciferase expression, in accordance with a very low luciferase level in the parental population. Fig. 4B shows the analysis of individual clones that were not subcloned from preselected populations but isolated as individual colonies after the initial G418 selection in two infection experiments with low IIR (0.005% and 0.7%) and one experiment with high IIR (22%). All three sets of clones showed a wide range of luciferase activity, but the luciferase levels in one half of the clones from the 22%-IIR infection were higher than in any of the clones from the low-IIR sets. Mass populations of infected cells were also derived by G418 selection (in parallel with the clones) from the infections with 0.7% and 22% IIR and maintained in tissue culture for the same time as the clones. While the luciferase activity of the 22%-IIR mass population closely matched the average for the individual clones selected in the same experiment, the luciferase level in the 0.7%-IIR mass population was lower than in any of the 13 clones isolated in parallel (Fig. 4B). The latter result suggests that the 0.7%-IIR population contained only a few clones with very low luciferase activity at the time of infection, but these clones had a growth advantage in the mass population.

Effects of G418 Selection on the Expression and Stability of Reporter Gene Expression. To determine whether G418 selection affected luciferase expression in the selected populations, we have measured the luciferase activity in 7 independently infected populations prior to the addition of G418. The expected luciferase activity in the infected cells was then estimated by dividing the measured luciferase levels by the IIR for the corresponding experiments, and this expected luciferase activity was compared with the actual activity measured in the same populations after G418 selection. Fig. 5 shows that the actual and expected activities were similar in the populations with 6% and 9% IIR, but the actual activity after G418 selection gradually decreased (relative to the expected level) in populations with lower IIR. Thus,

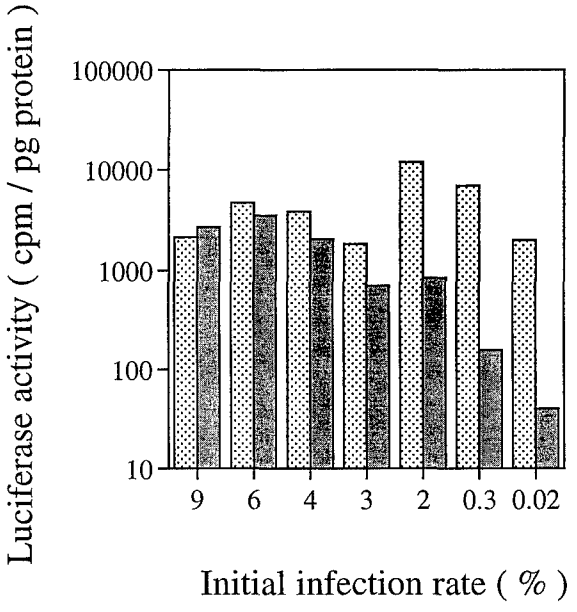


Fig. 5. Luciferase activity in HT1080 pJet-2fTGH cell populations before and after G418 selection. For each population (with the indicated IIR), the estimated luciferase activity of infected cells before G418 selection is shown on the left (light bars), and the actual luciferase activity measured 3–6 days after G418 selection is shown on the right (dark bars). The estimated activity before selection was determined by measuring the luciferase activity 3 days after the infection, prior to the addition of G418, and dividing this value by the IIR determined for the same infection.

G418 selection was detrimental to luciferase expression in populations with low IIR, but had no major effect on higher-IIR populations.

We have also analyzed the stability of luciferase expression in 11 G418-selected populations with IIR ranging from 0.1% to 33%. Cells were maintained in the presence or in the absence of G418 up to 76 days after completion of the initial G418 selection (defined as the time when no viable cells remained in uninfected control plates), and luciferase levels were measured on days 12, 19, 36, and 76 (Fig. 6). When maintained in the absence of G418, 8 populations with 1%–33% IIR showed no significant change in luciferase expression between days 12 and 36, though by day 76 these populations showed a moderate (11%–54%) decrease relative to day 12. In contrast, three populations with very low IIR (0.1%, 0.2%, and 0.6%) showed continuous loss of luciferase expression in the absence of G418, with 83%–97% of activity lost by day 76. Since the latter populations included a high proportion of cells with no luciferase expression (see Fig. 4A for clonal analysis of the 0.2%-IIR population), this loss can be explained by random "drift" that occured during the passaging of cells, rather than intrinsic instability of luciferase expression in the infected cells. In agreement with this interpretation, two luciferase-expressing clones

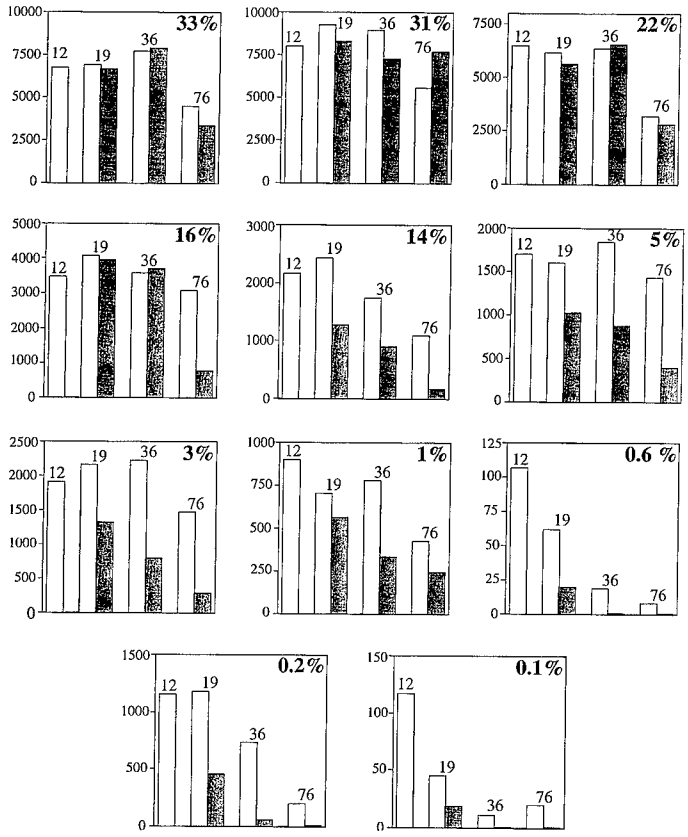


Fig. 6. Stability of luciferase expression in G418 selected populations of HT1080 pJet-2fTGH cells. The IIR values for the corresponding populations are indicated in boldface. The Y axis represents luciferase activity (in cpm/pg protein) for the corresponding cell populations, measured on days 12, 19, 36, and 76 after the completion of G418 selection, as indicated by numbers on top of the bars. Light and dark bars correspond to cell populations maintained in the absence or in the presence of G418, respectively.

isolated from the 0.2%-IIR population showed no decrease in luciferase activity over a period of 45 days after subcloning (data not shown).

Parallel cell cultures, maintained in the presence of G418, showed much more rapid loss of luciferase expression in most of the populations, except for three populations with the highest IIR (22%, 31%, and 33%). In particular, three populations with the lowest IIR values lost their luciferase activity completely or nearly completely by day 36, when grown in the presence of G418 (Fig. 6). This result further indicates that exposure of low-IIR populations to G418 provides a selective advantage for cells with very low luciferase activity.

Lack of Reporter Gene Expression in Low IIR Populations is Associated with Proviral DNA Rearrangements. To determine if differences in luciferase expression reflected changes in the copy number or structural integrity of integrated proviruses, Southern blot analysis was carried out on genomic DNA from different infected populations or clones. Two types of assays were used (Fig. 1). In the first assay, genomic DNA was digested with *Eco*RI, which cuts twice within LNCluc, excising an internal 2.6 kb fragment that contains the coding sequence of *neo,* the internal CMV promoter and the 5′ part of *luc.* A probe corresponding to this fragment would hybridize with the same 2.6 kb band for each integrated provirus (unless proviral DNA is rearranged), with the band intensity proportional to the provirus copy number. In the second assay, genomic DNA was digested with *Hind*III, which cuts once in the LNCluc provirus, between the CMV promoter and the luciferase gene. A probe corresponding to the 2.0 kb *Eco*RI–*Hind*III fragment of LNCluc would hybridize with different bands for each integrated provirus, since the size of each band would be determined by the location of a HindIII site in the flanking cellular sequences (Fig. 1). Thus, the number of different hybridizing fragments in the *Hind*III digest would correspond, in the first approximation, to the number of different integration events (as limited by the resolution of Southern blots).

We have applied this analysis to a low (0.2%) IIR population and cellular clones derived from it. *Eco*RI analysis of 10 clones from this population (Fig. 7A, lanes 1–10) revealed that only one of them, clone 2 showed the expected single band of 2.6 kb size (lane 2). The other clones showed instead a major band of approximately 3.5 kb size, suggesting a common rearrangement in the provirus carried by these clones (the increased size of the band indicates the loss of the 3′ internal *Eco*RI site). This conclusion was confirmed by *Hind*III analysis (Fig. 7B, lanes 2–7), where each of the

Fig. 7. Southern hybridization analysis of HT1080 cell clones and populations infected with LNCluc retrovirus. Except where indicated, infected cells were derived from HT1080 pJet-2fTGH cell line. Genomic DNA (10 mg) was digested with *Eco*RI (panels A, C, and D) or *Hind*III (Panel B). *Eco*RI digests were hybridized with a 2.6 kb probe from LNCluc (Fig. 1) (above) and subsequently with a control pMDR2 probe (below). HindIII digests were hybridized with a 2.0 kb probe from LNCluc (Fig. 1). The sizes of the bands mentioned in the text are indicated. Individual lanes contain DNA from the following sources: (A) 1–10, clones 1–10 (respectively) from 0.2% IIR population; 11–20, clones 1–10 (respectively) from 31% IIR population. (B) 1, 0.2% IIR population; 2–7, clones 1, 2, 4, 5, 9, and 10 (respectively) from 0.2% IIR population; 8–13, clones 1, 2, 3, 6, 7, and 8 (respectively) from 31% IIR population; 14, 31% IIR population. (C) 1, uninfected HT1080 cells; 2, 0.2% IIR population; 3, 0.2% IIR population, cultured for 76 days in the presence of G418; 4 and 5, clones 1 and 2 (respectively) from 0.2% IIR population; 6, 0.7% IIR population; 7, 31% IIR population; 8, 31% IIR population, cultured for 76 days in the presence of G418; 9 and 10, clones 1 and 8 (respectively) from 31% IIR population; 11, 16% IIR population; 12, 16% IIR population, cultured for 76 days in the presence of G418; 13, 22% IIR population. (D) 1, uninfected HT1080 cells (original line); 2, 0.02% IIR population (derived from original HT1080 cell line); 3–6, clones 1, 2, 3, and 4 (respectively) from 0.02% IIR population; 7, 6% IIR population (infection of original HT1080 cell line); 8–11, clones 1, 2, 3, and 4 (respectively) from 6% IIR population; 12, clone 1 from 0.2% IIR population; 13, clone 8 from 31% IIR population; 14, cell population infected on day 1 (9% IIR) and selected with 0.4 mg/ml G418; 15, cell population infected on day 1 plus day 2 (6% IIR) and selected with 0.4 mg/ml G418; 16, cell population infected on day 1 and selected with 2.5 mg/ml G418; 17, cell population infected on day 1 plus day 2 and selected with 2.5 mg/ml G418; 18, cell population infected on day 1 and selected with 5 mg/ml G418; 19, cell population infected on day 1 plus day 2 and selected with 5 mg/ml G418;

tested clones showed one 11 kb band, indicative of a single provirus integration. This band was seen in all the clones other than clone 2 which instead contained a 8.4 kb band (lane 3 in Fig. 7B), indicating that all the clones with the rearranged provirus had a common origin. *Hind*III and *Eco*RI analysis of the DNA from the parental 0.2%-IIR cell population shows that the

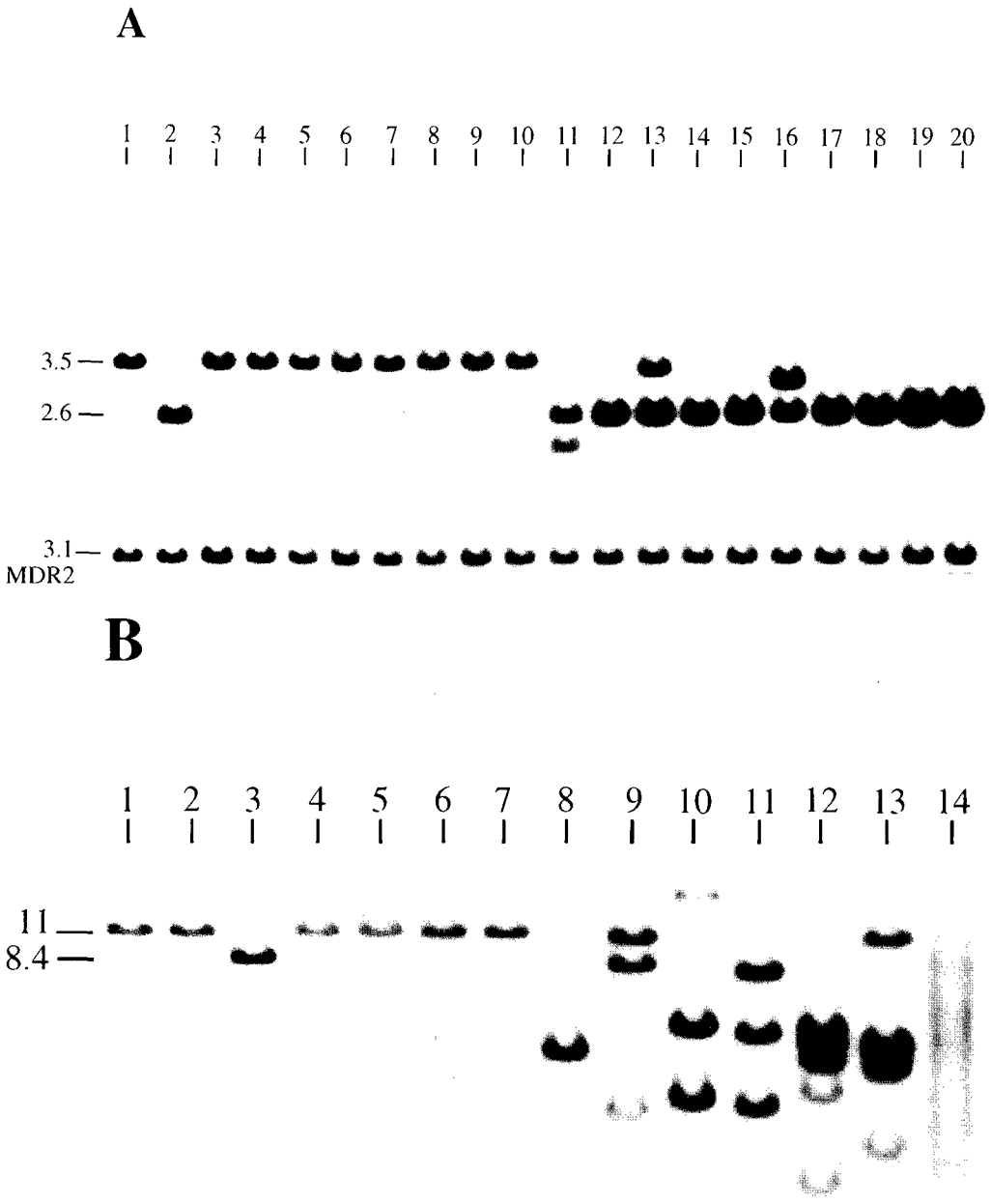
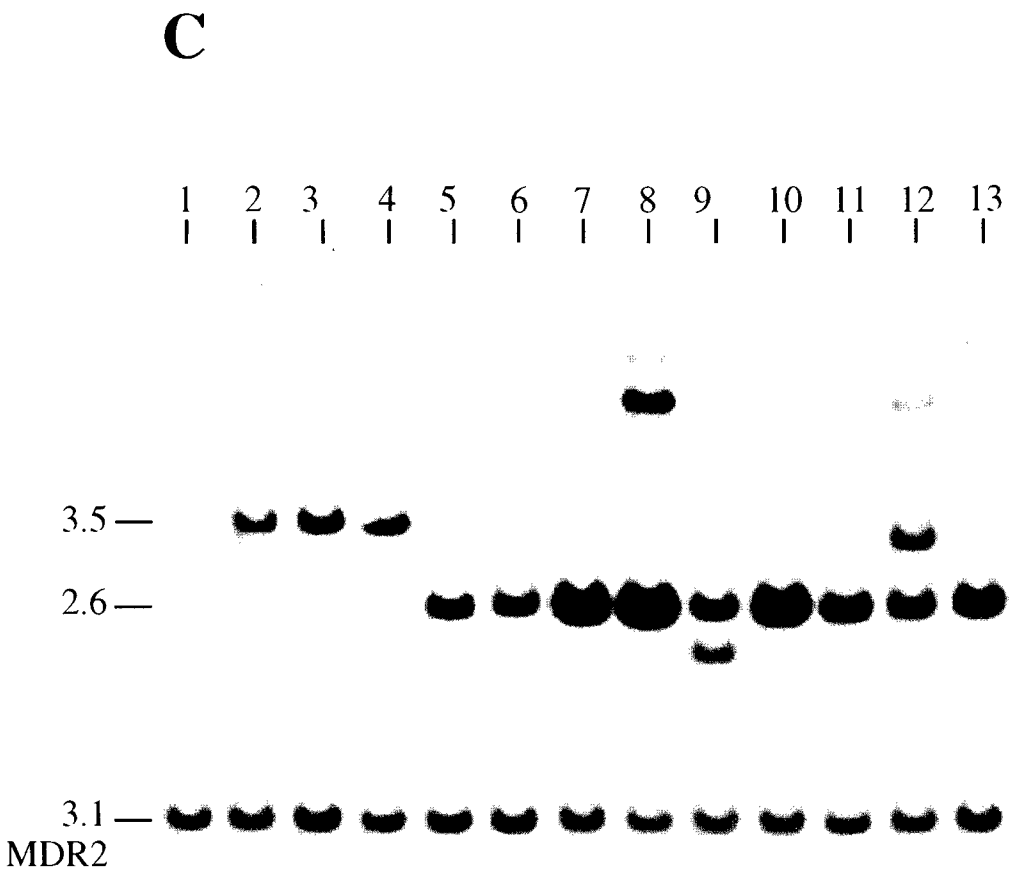


Fig. 7.



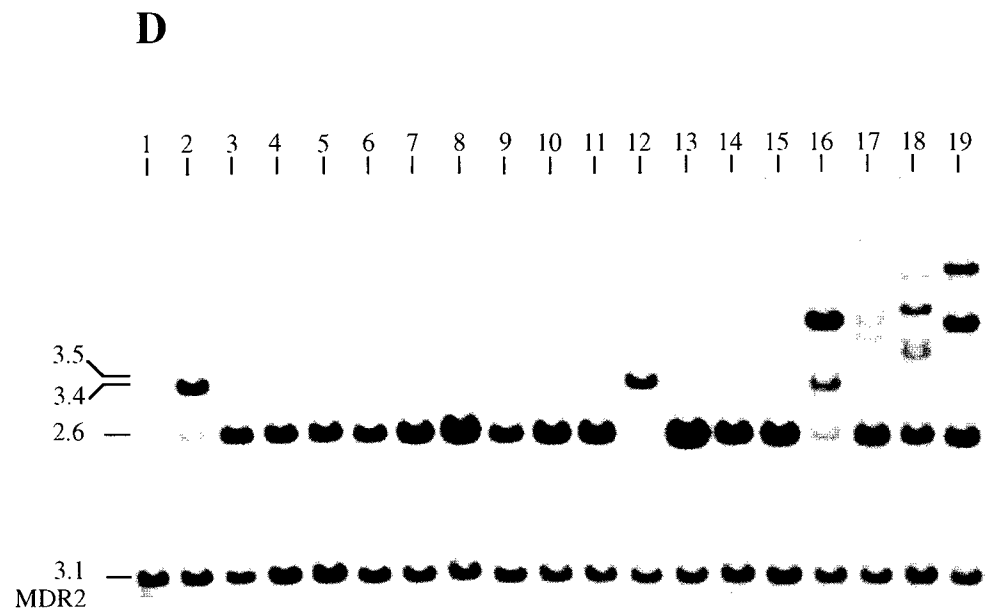


Fig. 7. (Continued)

bands corresponding to the rearranged provirus were predominant in this population prior to cloning (lane 1 in Fig. 7B and lane 2 in Fig. 7C). The 0.2%-IIR population, grown for 76 days after the removal of G418 still contains the unrearranged 2.6 kb band as a minor component (lane 2 in Fig. 7C), but this band is undetectable in cells maintained for the same time in the presence of G418 (lane 3 in Fig. 7C).

The luciferase activity of the clones derived from the 0.2%-IIR population is illustrated in Fig. 4A. Only clone 2, which shows no provirus rearrangement, possessed significant luciferase activity. The other clones were completely negative, except for clone 5 which showed very low luciferase activity. *Eco*RI analysis of this clone (lane 5 in Fig. 7A) shows that clone 5 contains a weak band corresponding to the unrearranged 2.6 kb fragment, in addition to the major 3.5 kb rearranged band. This clone therefore appears to be not a pure cell line but rather an unequal mixture of two cellular clones. PCR analysis of genomic DNA from the rearranged and unrearranged clones derived from the 0.2%-IIR population showed that the rearrangement, while leaving the *neo* gene intact, involves a complete or partial loss of the CMV promoter (data not shown), thus accounting for the lack of luciferase expression from the rearranged provirus. Thus, G418 selection of the 0.2%-IIR population has enriched for a clone containing a specific DNA rearrangement that abolishes luciferase expression from the integrated provirus.

In contrast to the 0.2%-IIR population, another low (0.7%) IIR population was found by *Eco*RI analysis to contain primarily unrearranged provirus, though a minor subpopulation with a specific rearrangement was also detectable in these cells (lane 6 in Fig. 7C); the 0.7%-IIR population also showed higher luciferase expression than the 0.2%-IIR population. We have also analyzed a very low (0.02%) IIR population, which was obtained after infection of the original HT1080 cell line (without ecotropic receptor) with amphotropic LNCluc virus, derived by transient transfection of BING packaging cells (21). As shown in Fig. 7D (lane 2), this population, like the 0.2%-IIR population, contained a single predominant rearranged band (3.4 kb size), which was more intense than the unrearranged 2.6 kb band present in the same population. Lanes 3–6 in Fig. 7D correspond to 4 clones that were selected with G418 as individual colonies after the same 0.02%-IIR infection. In contrast to the population, these clones showed only the unrearranged 2.6 kb band. Two of these clones expressed detectable luciferase levels, while 2 other clones did not express luciferase at all despite the apparently intact provirus. Thus, loss of expression from an internal promoter in our system does not necessarily involve a gross DNA rearrangement.

Reporter Gene Expression in High-IIR Populations Correlates with the Copy Number of Unrearranged Proviruses. *Eco*RI analysis of 10 clones derived from a high (31%) IIR population (lanes 11–20 in Fig. 7A) showed that all of these clones contained the unrearranged 2.6 kb band, but three of the clones (lanes 11, 13, 16) contained additional rearranged bands of different sizes. The uncloned 31%-IIR population also showed the predominance of the unrearranged band (lane 7 in Fig. 7C), though growth of this population for 76 days in the presence of G418 led to the appearance of prominent secondary bands corresponding to rearranged proviruses (lane 8 in Fig. 7C). Furthermore, quantitative analysis of signal intensity of the 2.6 kb band (using a probe corresponding to a fragment of the MDR2 gene as a normalization control) indicated that the unrearranged provirus was present at an average of 4.4 copies per cell in the 31%-IIR population and at different copy numbers (1–9 copies per cell) in individual clones. These conclusions were confirmed by *Hind*III analysis (lanes 8–14 in Fig. 7B), which showed a continuous smear (indicative of a very large number of provirus integration sites) in the uncloned 31%-IIR population (lane 14), and multiple discrete bands in most of the tested clones from this population (lanes 9–13).

*Eco*RI analysis of other populations with

high IIR values showed that the presence of multiple copies of unrearranged provirus is common in such populations. Thus, a 16%-IIR population (lane 11 in Fig. 7C) contained an average of 2.5 copies of unrearranged provirus per cell, with no detectable rearrangements. As with the 31%-IIR population, prominent rearranged bands became apparent in this population after continuous cultivation in the presence of G418 (lane 12 in Fig. 7C). Similarly, a 22%-IIR population (lane 13 in Fig. 7C) contained an average of 1.9 provirus copies per cell, without major rearrangements. Surprisingly, two populations with IIR as low as 6% contained an average of 2.4 or 2.6 provirus copies per cell, indicating that multicopy integration does not require high infection efficiency. One of these 6%-IIR populations (obtained after amphotropic virus infection of the original HT1080 cells) is shown in lane 7 of Fig. 7D (2.6 copies per cell), while 4 clones isolated in parallel with this population (lanes 8–11 in Fig. 7D) contained 1.5–5.2 copies per cell. As shown in Fig. 8, there is a general (though imperfect) correlation between IIR and the copy number of integrated unrearranged proviruses in 9 tested populations ($r^2 = 0.654$).

Fig. 9 shows a comparison between the copy number of unrearranged proviruses and the luciferase activity in 5 populations and 9 clones of HT1080 pJet-2fTGH cells that contained more than one unrearranged provirus per cell. Luciferase expression was strongly correlated with the copy number of unrearranged proviruses ($r^2 = 0.879$), indicating that the provirus copy number is the primary determinant of luciferase expression in high-IIR populations. It should be noted, however, that LNCluc-infected clones of the original HT1080 cell line expressed 2–3 times less luciferase per provirus copy than did the cells of the pJet-2fTGH subline (data not shown), indicating the importance of cell-specific factors in gene expression from retroviral vectors.

Effects of Increased Stringency of G418 Selection on Luciferase Expression and Provirus Rearrangements. The results described above indicate that G418 selection of infected populations under standard conditions (0.4 mg/ml) is detrimental to the expression from the internal CMV promoter in low-IIR populations. In populations with IIR ≥ 6%, however, G418 selection had no major effect on luciferase expression, though prolonged exposure of such populations to G418 caused a noticeable decrease in luciferase expression and

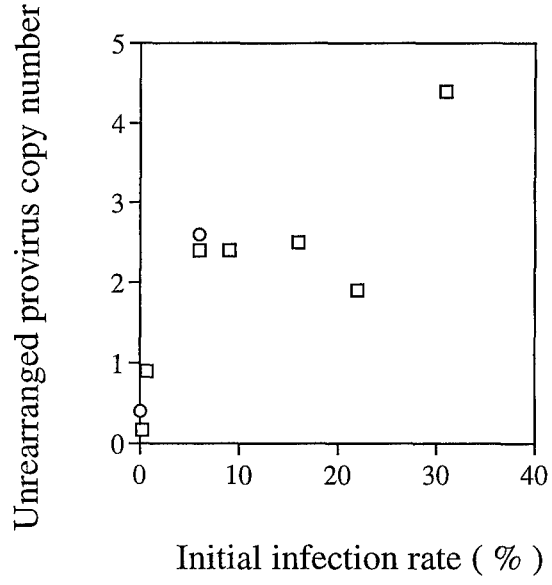


Fig. 8. Relationship between IIR and the average unrearranged provirus copy number in LNCluc-infected mass populations. Populations derived from HT1080 pJet-2fTGH subline are indicated with squares and populations from the original HT1080 line are indicated with circles.

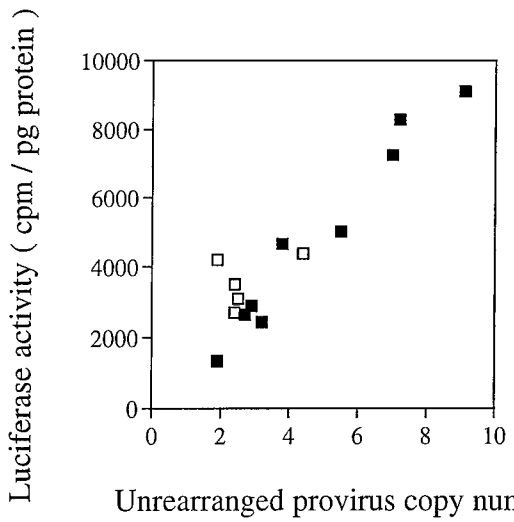


Fig. 9. Relationship between unrearranged provirus copy number and luciferase expression in different clones and populations of LNCluc-infected HT1080 pJet-2fTGH cells. Cell populations are indicated with open squares and clones with closed squares.

emergence of cell subpopulations with rearranged proviruses.

To determine how luciferase expression would be affected by increasing the concentration of G418 used in the original selection, LNCluc virus, derived by BOSC 23 transfection, was used to infect recipient cells either once (1 day or 2 days post-transfection) or twice (on days 1 and 2). The infected cells were then selected at a range of different G418 concentrations, from the standard 0.4 mg/ml up to 5 mg/ml G418. The IIR values, as determined at 0.4 mg/ml G418, were 9% for cells infected on day 1, 4% for cells infected on day 2, and 6% for cells infected on day 1 and day 2. As expected, the survival fraction in all three infected populations decreased with increasing concentrations of G418 (data not shown).

The luciferase activity of populations selected at different G418 concentrations showed an overall decrease with increasing G418 concentrations, but the extent of the decrease varied among the three populations (Fig. 10A). While the population infected on day 2 showed a complete loss of luciferase expression at the highest G418 concentration (5 mg/ml), the

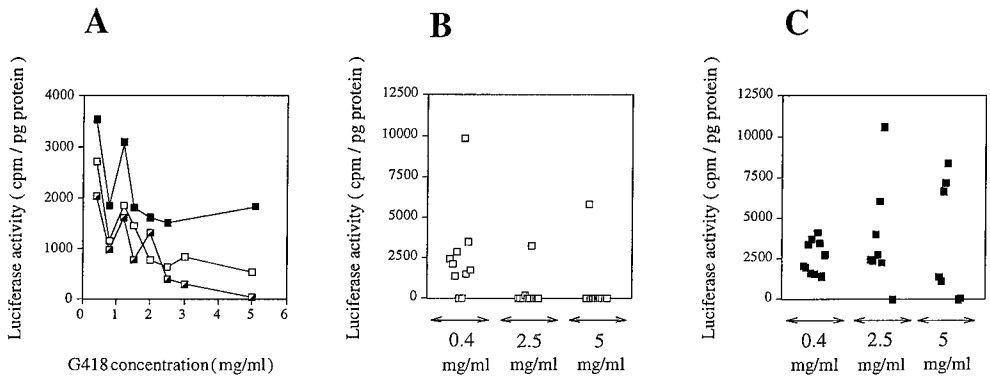


Fig. 10. Luciferase expression in LNCluc-infected HT1080 pJet-2fTGH cells, selected at different G418 concentrations. (A) Relationship between selective concentration of G418 (mg/ml) and luciferase activity in cell populations infected on day 1 (open squares), day 2 (half-open squares), and on day 1 plus day 2 (closed squares) after BOSC 23 transfection. Luciferase activity was determined 5 days after the completion of G418 selection. (B and C) Luciferase expression in clones isolated from the populations infected on day 1 (B) or on day 1 plus day 2 (C) and selected with the indicated G418 concentrations.

luciferase levels in the populations infected on day 1 and selected with 2.5–5 mg/ml G418 stabilized at the plateau corresponding to approximately 20% of the expression observed at 0.4 mg/ml. The decrease was much less pronounced in the doubly-infected population, where luciferase expression at G418 concentrations from 1.5 to 5 mg/ml showed a plateau corresponding to approximately 50% of the level obtained at 0.4 mg/ml.

An interesting picture emerged from the analysis of luciferase expression in cell lines subcloned (in the absence of G418) from the populations that were infected on day 1 or on day 1 plus day 2 and selected at 0.4, 2.5, or 5 mg/ml G418 (Fig. 10B,C). Most of the clones isolated from both populations selected at 0.4 mg/ml showed intermediate levels of luciferase expression. In contrast, almost all the clones from the singly-infected population were luciferase-negative when selected at 2.5 or 5 mg/ml G418 (Fig. 10B). Increasing G418 concentration, however, had a different effect on doubly-infected cells (Fig. 10C). At 2.5 mg/ml, 5 of 8 clones in this group showed intermediate luciferase activity, but 2 clones showed high levels of luciferase and one clone was negative. At 5 mg/ml, 2 of 7 clones became luciferase-negative, 2 clones showed low luciferase activity, and 3 clones expressed a high level of luciferase. This analysis suggests that increasing concentrations of G418 may select for both luciferase-negative clones and for clones expressing higher than average levels of luciferase.

*Eco*RI Southern hybridization analysis was carried out on the populations infected on day 1 and on day 1 plus day 2 and selected at 0.4, 2.5, or 5 mg/ml G418 (lanes 14–19 in Fig. 7D). Both of the populations selected in 0.4 mg/ml G418 contained primarily the unrearranged (2.6 kb) band, with the intensity corresponding to 2.4 copies per cell in both cases (lanes 14, 15). In contrast, populations selected at 2.5 or 5 mg/ml G418 developed prominent rearranged bands (lanes 16–19). The appearance of the rearranged bands and, in some cases, a decrease in the intensity of the apparently unrearranged band, paralleled the observed decrease in the luciferase activity of the corresponding populations.

DISCUSSION

In the present study, we have analyzed the effect of differences in the infection rate and G418 selection conditions on the expression of a reporter gene (luciferase) from an internal promoter of a double-gene retroviral vector, LNCX, transduced into human HT1080 fibrosarcoma cells. Our study was deliberately limited to a single recipient cell/vector combination, since we wanted to use a clearly defined experimental system to identify the factors responsible for experimental variability in the expression of retrovirally transduced genes. Nevertheless, the types of problems that were identified in our study (instability of gene expression from retroviral vectors, poor expression of the nonselected gene, proviral DNA rearrangements) have been commonly observed with many different recipient cell types and with other classes of double-gene retroviral vectors. We believe therefore that the general correlations observed in this work will be applicable in principle to other cell/vector systems, and should be considered in designing functional assays for genes or genetic elements transduced by retroviral vectors.

The first non-obvious result of our study is that different retrovirally infected cell populations, selected with G418 and therefore consisting entirely of transduced cells, showed widely varying levels of luciferase expression, and in some cases did not express luciferase at all. Also unexpectedly, these differences turned out to correlate with the IIR of the tested populations. In particular, all the populations with very low or no luciferase expression arose from the infections with IIR $< 1\%$. Furthermore, populations with IIR $\leq 5\%$ showed lower luciferase expression than the populations with IIR $\geq 6\%$, and the levels of expression in the latter group were significantly correlated with their IIR values. Thus, IIR determination can be used to identify populations that may fail to express the

nonselected gene, and to gauge the likely relative levels of gene expression among independently infected populations. We would expect, however, that the quantitative parameters of the correlation between IIR and gene expression would be different for different cell/vector systems, as we have observed even for two different sublines of HT1080 cells infected with the same retroviral vector.

G418-selected populations with low IIR, as well as clones derived from such populations, showed single-copy provirus integration, with a high incidence of proviral DNA rearrangements. Provirus rearrangements in double gene vectors have been previously observed by other investigators (7, 12). These rearrangements affect the transcription unit driven by the internal promoter and therefore abolish reporter gene expression. On the other hand, some of the clones that did not express luciferase showed apparently intact proviral DNA, suggesting that the internal promoter in these clones was inactivated without a major rearrangement, as previously observed in other cell/vector systems (5, 7). Clonal analysis of low-IIR populations demonstrated that they generally consist of two types of cells: those that do not express luciferase at all, and those that contain a single copy of an unrearranged provirus and express luciferase at an intermediate level. The levels of luciferase expression, which vary widely among low-IIR populations appear to be determined primarily by the ratios of these two cell types within each population. As will be discussed elsewhere in this section, the loss of luciferase expression in low-IIR populations is a consequence of G418 selection.

Analysis of genomic DNA from the high-IIR populations demonstrated that all the tested populations with IIR between 6% and 31% contained from 2 to 4.4 copies of integrated unrearranged provirus per cell. The provirus copy number in different populations showed some correlation with IIR. More importantly, the levels of luciferase expression among the high-IIR populations and the corresponding clones were strongly correlated with the copy number of unrearranged proviruses. This suggests that the copy number is the primary determinant of gene expression in these populations, or in single clones containing multiple integrated proviruses. On the other hand, such factors as the provirus integration site are likely to be responsible for the variability in gene expression among cell clones containing a single copy of unrearranged provirus.

Multicopy integration of retroviral vectors has been previously demonstrated not only at the level of individual clones, but also in the total infected populations. In such populations, however, the observed infection rates were 80% (7) or 100% (14). In contrast, we have detected multicopy integration at infection rates as low as 6% and at MOI as low as 0.5 (as calculated for a 6%-IIR population of HT1080 cells that were infected with amphotropic virus and acquired an average of 2.6 proviruses per cell). Multicopy integration in such populations most probably is not a consequence of preferential G418 selection of cells with more than one provirus, since in such a case one could expect the luciferase levels to be increased in G418-selected relative to unselected populations. However, luciferase levels measured before and after G418 selection in two independent populations that acquired 2.4 provirus copies per cell were in close agreement. Multicopy integration occurring at infection rates that are much lower than 100% suggests that the recipient cells contain a subpopulation which is preferentially susceptible to retroviral infection, and which is likely to acquire two or more proviruses while the bulk of the population remains uninfected. The remaining cells, however, are not entirely refractory to retroviral infection, since up to 78% of the recipient cells could be infected at higher titers of LNCluc. Retroviral infectability has been related to the distribution and interactions of viral receptors on the cell surface (30, 31), metabolic factors affecting reverse transcription (32, 33) or provirus integration (34), the ability of cells to divide (35), and phase of the cell cycle (36).

G418 selection, however, appears to play a

critical role in enriching for cells that have lost the internal promoter function. Thus, the luciferase levels measured after G418 selection of cell populations with IIR < 2–3% were much lower than the levels calculated from the luciferase measurements conducted before G418 selection. While G418 selection under standard conditions showed no significant effect on cell populations with higher IIR, prolonged growth of such populations in the presence of G418 led to a partial loss of luciferase expression and an emergence of rearranged proviral sequences, in agreement with the previous observation of Olsen et al. (12). An increase in the G418 concentration used for the initial selection of populations with higher IIR had a dual effect. Higher G418 concentrations increased the proportion of cells that have lost luciferase expression, which was accompanied by an increase in proviral DNA rearrangements. This negative effect of high G418 concentrations appears to be partially offset, however, by concurrent selection of a subpopulation of cells with increased luciferase expression, presumably reflecting a higher copy number of unrearranged proviruses in such cells.

The observed differential effects of G418 selection on luciferase expression in populations with different IIR can be explained by considering the nature of G418 selection. The drug G418 (a translation inhibitor) induces either complete or partial growth inhibition in mammalian cells, depending on the intracellular drug concentration and the intrinsic cellular sensitivity to this drug. The bacterial *neo* gene encodes neomycin phosphotransferase, an enzyme that metabolically inactivates G418, with the extent of inactivation dependent on the drug concentration and on the amount of enzyme produced by *neo*-expressing cells. Under our empirically determined selection conditions (0.4 mg/ml G418), there is no survival of HT1080 cells that do not contain *neo,* but cells carrying at least one copy of a *neo*-expressing provirus are able to grow. However, cells that express an elevated level of neomycin phosphotransferase (either through the presence of more than one copy of *neo* or through some changes increasing the expression of the single *neo* gene) are apt to have a growth advantage in the presence of the drug, as they inactivate G418 more efficiently (9). Since two adjacent promoters present in the same vector can interfere with each other (5), a rare event silencing the internal CMV promoter (through proviral DNA rearrangement or some other mechanism) is likely to increase the expression of the *neo* gene and therefore provide cells with a growth advantage in the presence of G418. As a result, such cells become enriched after G418 selection of low-IIR populations (where multicopy integration is very rare). In contrast to the low-IIR populations, most of the cells infected at high IIR contain two or more copies of the provirus, which provides them with an adequate level of *neo* expression for optimal growth. As a result, cells with inactivation of the internal promoter do not show significant enrichment in high-IIR populations under standard selection conditions. Nevertheless, such cells gain a selective advantage with an increase in the stringency or the duration of G418 selection, as has been observed in our study.

It is conceivable that some of the problems that we have characterized could be diminished or obviated through changes in the vector design. An attractive alternative to the selectable drug-resistance genes is provided by fluorescent markers, such as the green fluorescent protein (37) or physiologically neutral cell surface antigens detectable by immunofluorescence (7). Retrovirally transduced cells expressing such markers can be isolated by fluorescence-activated cell sorting shortly after infection, and no continuous selection would be required for these markers. Another general change would be in the relative position of the two genes. Some investigators have reported improved expression of the nonselected gene when such a gene is expressed from the LTR and the selected gene from an internal promoter (7, 8, 13), though others have reported the problems of poor expression, rearrangement or instability with such vectors as well (5, 7). In our hands, a vector

of this type, LXSN (20), does not appear to provide an improvement over LNCX in several tested cell lines, but we have not analyzed the LXSN vector systematically. Finally, vectors expressing both genes from a single bicistronic message, using an IRES for translation initiation of the second gene, would in principle avoid the problem of "promoter competition" altogether, and they have in fact been reported to provide improved coexpression of the two genes relative to two-promoter vectors (3, 38). Due to the relative novelty of such vectors, however, it is still unclear how they compare to conventional vectors in the expression stability or viral titers. At any rate, double-gene vectors with the gene of interest transcribed from an internal promoter, still remain the primary choice in the cases where the LTR promoter does not provide adequate expression in the target cell type, or when the internal promoter is chosen for tissue-specific or inducible expression.

Based on the results of the present study, some general recommendations can be made for the functional analysis of genes transduced with double-gene retroviral vectors. First of all, one should keep in mind the possibility that some mass populations transduced with such a vector and selected for the function of one of the genes, may express the nonselected gene poorly or not at all, even if the same cell/vector combination has produced adequate expression in previous experiments. To avoid this problem, an ideal solution in theory would be to carry out the infection at a very high virus titer or in multiple rounds, so as to infect all the recipient cells and avoid selection altogether. Unfortunately, this solution is not feasible for most of the recipient cell types. Given the necessity of selection, it is important to maximize the virus titer so as to achieve an infection rate high enough to produce an average of more than one copy of integrated provirus per infected cell (as can be determined by DNA hybridization). Our results indicate that multicopy integration (at least in HT1080 cells) can be achieved with either ecotropic or amphotropic retrovirus even when the infection rate is relatively low. Furthermore, it would be helpful to minimize the stringency and the duration of selection for the selectable gene of the retroviral vector. When a cytotoxic drug (such as G418) is used for selection, the lowest dose and time of exposure sufficient to kill the uninfected cells should be determined for each recipient cell line, and the stringency of selection should not be increased. Finally, we would recommend assaying the RNA (or protein) expression of the nonselected gene in each transduced population, to assure consistent interpretation of different infection experiments.

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Inducible retroviral vectors regulated by *lac* repressor in mammalian cells

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Abstract

We have developed *lac* repressor-regulated retroviral expression vectors that are induced by β-galactosides upon stable transduction in mammalian cells. These vectors, derived from a Moloney-virus-based vector LNCX, contain an internal Rous sarcoma virus (RSV) or cytomegalovirus (CMV) promoter, coupled with 2 to 4 *lac* operator sequences and placed in *anti* orientation relative to the retroviral long terminal repeat (LTR). Three different vectors were tested in stably infected mass populations of mouse and human cells expressing the *lac* repressor, in parallel with the constitutively expressed LNCX vector. The highest expression levels from these vectors ranged from 1–4% to 25–33% of the LNCX level, and the induction by β-galactosides ranged from 6–11-fold to 29–54-fold. These vectors should be suitable for studies requiring efficient gene transfer and regulated expression in mass populations of stably transduced mammalian cells.

Keywords: Gene transfer; Promoter regulation; β-Galactosides; Luciferase; Retroviral integration

1. Introduction

Retroviral vectors provide one of the most efficient means for gene delivery and expression in a wide variety of mammalian cell types. In particular, the ability of these vectors to transduce up to 100% of recipient cells has been utilized for expression cloning and for testing the phenotypic effects of transduced genes on mass populations of cells rather than on subcloned transductants (Gudkov et al., 1993, 1994; Rayner and Gonda, 1994). The range of research applications for retroviral vectors could be significantly expanded by the ability to regulate gene expression within integrated proviruses, using physiologically neutral inducing agents. Such agents include β-galactosides or tetracycline, which affect promoter binding of modified repressor proteins from the *lac* (Hu and Davidson, 1987; Labow et al., 1990) or *tet* (Gossen and Bujard, 1992; Kim et al., 1995) operons of *E. coli*. Very recently, Paulus et al. (1996) described retroviral vectors regulated by a tetracycline-inhibited transactivator protein, but regulation of these vectors in established populations of stably infected cells has not been evaluated. In the present study, we have analyzed the ability of *lac* repressor (LacI)-based activator and repressor proteins to regulate the expression of *lac* operator-containing promoters in retroviral vectors integrated in mammalian cells.

2. Experimental and discussion

We have constructed and tested a series of β-galactoside regulated vectors, derived from the retroviral vector LNCX (Miller and Rosman, 1989) which expresses *neo* (G418 resistance) gene from the MoMLV/MoMSV LTR promoter and carries an internal CMV promoter for cloned gene expression (Fig. 1). The CMV promoter of LNCX was replaced with a β-galactoside regulated promoter in either *syn* or *anti* orientation relative to MoMLV LTR. For expression assays, the firefly luciferase reporter gene (*luc*) was inserted into each vector downstream of the regulated promoter.

We have initially used LAP267 and LAP348 transactivator proteins, which combine the DNA-binding domain of *E. coli* LacI repressor and a transcription activator domain of the herpes simplex virus type 1 protein VP16

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Abbreviations: CMV, cytomegalovirus; IPTG, isopropyl β-thiogalactoside; LacI, *lac* repressor; LAP, *lac* activator protein; *luc*, luciferase gene; LTR, long terminal repeat; MoMLV, Moloney murine leukemia virus; MoMSV, Moloney murine sarcoma virus; MTG, methyl β-thiogalactoside; RSV, Rous sarcoma virus; tc, tetracycline.

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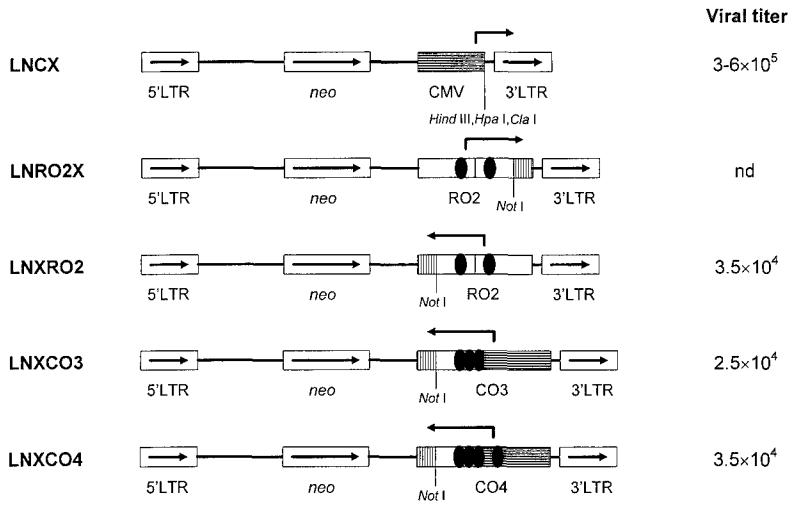

Fig. 1. Structure of retroviral vectors. Arrows indicate the direction of transcription. Unique restriction sites for insertion cloning are indicated. The promoters are: LTR, MoMLV LTR; CMV, CMV early promoter (horizontally striped box); RO2, a tripartite unit consisting of RSV LTR promoter (stippled box), *lac* operators (ovals) inserted upstream and downstream of the transcription start site in SV40 intron sequence (open box), and polyA signal (vertically striped box); CO3, CMV early promoter with 3 *lac* operators downstream of the transcription start site; CO4, CMV early promoter with 3 *lac* operators downstream and 1 *lac* operator upstream of the transcription start site. The viral titers were determined in parallel after transient transfection of BOSC23 packaging cells.

Methods. *(a) Cloning.* The details of the cloning procedures will be provided upon request. pLNCX (Miller and Rosman, 1989) was a gift of A.D. Miller (Fred Hutchison Cancer Center). RO2 was derived from pOPRSVICAT plasmid (Stratagene Cloning Systems). CO3 was derived by replacing the RSV promoter of the plasmid pOPI3CAT (Stratagene Cloning Systems) with the CMV promoter from pLNCX. CO4 was constructed by inserting a synthetic *lac* operator sequence at the *Alu*I site between the TATA box and the transcription start site of the CMV promoter from LNCX; this modified CMV promoter was then used to replace the RSV promoter of pOPI3CAT. For the expression assays, *luc* reporter gene from the plasmid pGEM*luc* (Promega Corporation) was inserted downstream of the corresponding promoters.

(b) Retroviral transduction. Ecotropic retrovirus-packaging cell line BOSC23 was a gift of W. Pear and D. Baltimore (Rockefeller University). Transient transfection of BOSC23 with retroviral vector plasmids was carried out as previously described (Pear et al., 1993). Forty-eight hours after transfection, serial dilutions of the media supernatants were used to infect NIH 3T3 cells in the presence of 4 μg/ml polybrene. Infected cells were selected with 0.4 mg/ml G418 for 8 days, and the colonies were scored after staining with crystal violet. Viral titer is expressed as colony forming units per ml of supernatant.

(Labow et al., 1990; Baim et al., 1991), to regulate *luc* expression from an enhancerless SV40 early promoter coupled with 21 *lac* operator repeats. When transiently transfected into mouse NIH 3T3 cells, both *syn* and *anti*-oriented vectors showed LAP-dependent expression, which was affected by isopropyl β-thiogalactoside (IPTG). However, when the same vectors were introduced into these cells by retroviral infection, stably infected cell populations showed equally high *luc* expression in the presence and in the absence of LAP activators (data not shown). This result, which can most readily be explained by preferential integration of proviruses in proximity to cellular enhancers (Sablitzky et al., 1993), suggested to us that transactivator-based regulation of enhancerless promoters may not be suitable for regulated gene expression in integrated retroviruses.

We have then investigated the ability of the LacI repressor, modified for nuclear localization in mammalian cells (Fieck et al., 1992), to regulate the expression of *lac* operator-containing complete promoters after retroviral transduction. Vectors LNRO2X and LNXRO2 were prepared by replacing the internal CMV promoter of the LNCX vector with the RSV LTR-derived promoter, modified by insertion of two *lac*

Table 1
Regulation of luciferase activity in mass populations of HT1080 or NIH3T3 cells transduced with *lac* repressor-regulated retroviral vectors

Vector	Fold induction[a]		Maximal expression (% of LNCX)[b]	
	HT1080	NIH3T3	HT1080	NIH3T3
LNXRO2	53.9±19.9 (15)[c]	28.9±10.1 (16)	1.0±0.6 (15)	3.5±0.9 (16)
LNXCO3	11.1±3.3 (15)	10.8±3.2 (14)	9.3±5.3 (15)	10.3±5.2 (14)
LNXCO4	5.6±1.4 (14)	10.5±2.6 (16)	33.1±12.5 (14)	24.6±7.6 (16)

[a] Fold induction is the ratio of luciferase activity in the presence of inducer (either IPTG or MTG) to the activity in the absence of the inducer.
[b] Maximal expression is the level of induced luciferase activity relative to the *luc*-expressing LNCX vector, assayed in parallel.
[c] Mean±SD; the total number of experiments is indicated in parentheses.

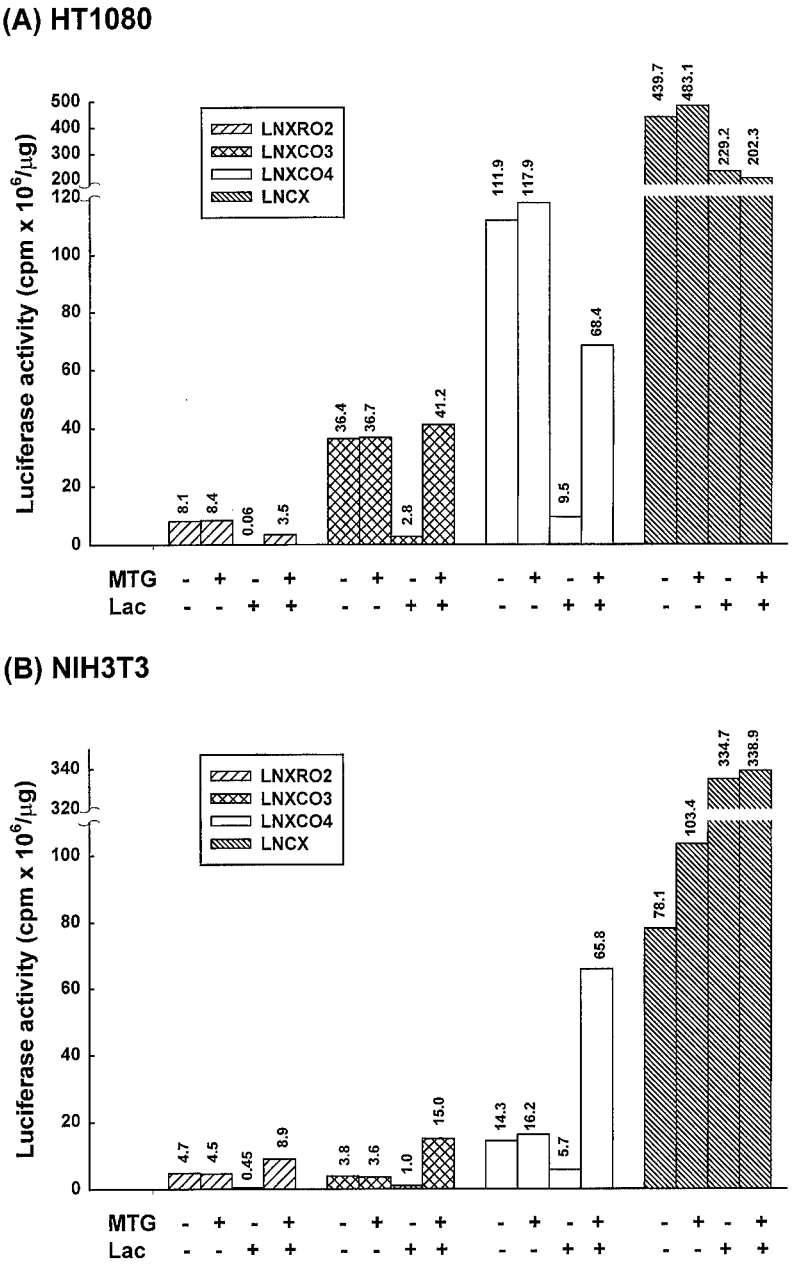

Fig. 2. Effects of *lac* repressor and MTG on luciferase production from the integrated LNXRO2, LNXCO3 and LNXCO4 vectors. Bars indicate luciferase activity of mass populations of human HT1080 (A) or mouse NIH 3T3 (B) cells, untransfected or stably transfected with the *lacI* gene and infected with the indicated *luc*-expressing retroviral vectors, as determined in the presence or in the absence of 5 mM MTG.

Methods. Plasmid p3′SS (Stratagene Cloning Systems), expressing a modified *lac* repressor and carrying a hygromycin-resistance gene, was transfected by standard calcium phosphate precipitation into HT1080 cells containing ecotropic retroviral receptor (Albritton et al., 1989; the cell line was generated by E. Kandel in our laboratory), or into NIH3T3 cells. Transfectants were selected with 100–120 μg/ml of hygromycin, and individual colonies were picked and expanded. Transfectant clones were screened for the optimal *lac* repressor activity in two steps. The first step involved transient co-transfection with equal amounts of plasmid pOPRSVI*luc* (generated by replacing CAT of pORRSVICAT with *luc*) which expresses *luc* from RO2 promoter, and control plasmid pSV2βgal (Promega Corporation) expressing β-gal from the SV40 promoter, in the presence or absence of 10 mM IPTG, and assayed for luciferase and β-galactosidase (normalization control) activities 48 h later. Seven of 13 tested HT1080 clones and 5 of 50 NIH 3T3 clones gave >40-fold increase in the normalized luciferase levels in the presence of IPTG. These clones were then tested for regulated *luc* expression after retroviral transduction with *luc*-carrying LNXRO2, as described below. Clones giving the highest fold induction in this second step of analysis were used in the present experiment.

Retroviral transduction and luciferase assays. Retroviral transduction was carried out as described in Fig. 1. Mass populations of infected HT1080 (A) or NIH3T3 (B) cells were selected with 0.4 mg/ml G418 for 8 days and expanded in the absence of the drug. Infections with different viruses were carried out in parallel, using all the retroviruses at similar titers. For luciferase assays, cells (10^5 cells per 3.5 cm well) were incubated for 24 h in the presence or absence of 10 mM IPTG. Luciferase activity was measured using the Luciferase Assay System (Promega Corporation) and normalized for the amount of cellular protein, as determined by modified Bradford method using a Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories).

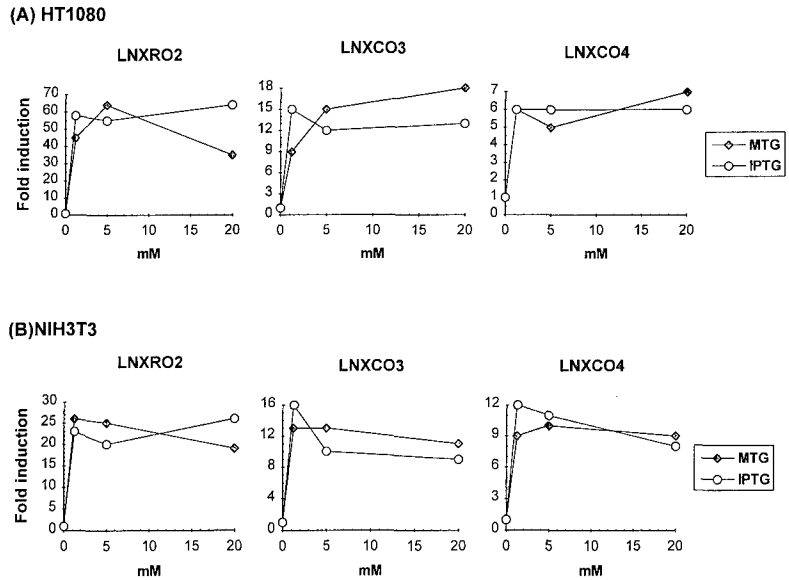

Fig. 3. Dependence of luciferase production from *lac* repressor-regulated retroviral vectors on IPTG and MTG concentrations. The assays on mass populations of *lacI*-expressing HT1080 (A) and NIH 3T3 (B) cells were carried out as described in Fig. 2, using different MTG or IPTG concentrations. Fold induction is the ratio of luciferase activity at a given concentration of the inducer to the activity in the absence of the inducer.

operator sequences upstream and downstream of the transcription start site, in either *syn* (LNRO2X) or *anti* (LNXRO2) orientation relative to MoMLV LTR (Fig. 1). During transient co-transfection of NIH 3T3 cells with a *lacI* expressing plasmid, both vectors, carrying the *luc* gene, showed 100- to 150-fold increase in luciferase activity in the presence of IPTG (data not shown). We then introduced these vectors into NIH 3T3 cells by retroviral transduction, following transient transfection of BOSC23 packaging cells (Pear et al., 1993). The recipient cells were either untransfected or stably transfected with the *lacI* expressing plasmid. The luciferase activity was measured in G418-selected mass populations of stably infected cells, in the presence or absence of IPTG. *luc* expression from the *anti*-oriented LNXRO2 vector was strongly inhibited by *lac* repressor in the absence but not in the presence of IPTG (see below), but the *syn*-oriented LNRO2X vector showed only minor (about 2-fold) decrease in *luc* expression in the presence of the repressor and only 2–3-fold induction by IPTG (data not shown). Thus, the *anti* orientation of the internal promoter is preferable for LacI regulation in an integrated retrovirus.

In addition to LNXRO2, we constructed two other *anti*-oriented vectors, where *lac* operators were inserted into the CMV rather than RSV promoter (Fig. 1). The LNXCO3 vector carries three *lac* operators downstream of the transcription start site, and LNXCO4 has an additional (fourth) *lac* operator upstream of the transcription start site. All three *anti*-oriented vectors, when transiently transfected into retrovirus-packaging cells, yielded similar retroviral titers, which were an order of magnitude lower than the titer of the virus derived from the highly efficient LNCX vector (Fig. 1). These three vectors, as well as LNCX, were supplied with the *luc* reporter gene and used for retroviral transduction into human HT1080 fibrosarcoma or mouse NIH 3T3 cell line. The recipient cell clones were derived after stable transfection with *lacI,* subcloning and screening of subclones for the ability to regulate reporter gene expression in transient transfection assays.

G418-selected mass populations of cells stably infected with different retroviruses (>10 000 clones in each population) were tested for luciferase activity, in the presence or in the absence of IPTG or methyl β-thiogalactoside (MTG). The results of these assays are summarized in Table 1, and luciferase activities from a representative set of assays (carried out in parallel) are shown in Fig. 2(A,B). In *lacI*-transfected populations of HT1080 and NIH 3T3 cells, the RSV-based LNXRO2 vector showed the highest-fold induction with MTG or IPTG, with an average of 54-fold in HT1080 and 29-fold in NIH 3T3. The expression levels in cells without *lacI* were similar to the levels observed in the presence of an inducer in *lacI*-expressing cells. The fully induced expression levels for LNXRO2, however, was on the average only 1% (for HT1080) or 4% (for NIH 3T3) of the expression provided by the constitutive LNCX vector in the same cells. In contrast, the CMV-based inducible vector LNXCO4 provided much higher maximal levels of expression, with the average of 33% (HT1080) or 25% (NIH 3T3) of the LNCX level. This vector, however, showed the average induction of only 6-fold (in HT1080) or 11-fold (in NIH 3T3). The other CMV-based vector, LNXCO3, showed an intermediate level

of maximal expression and inducibility relative to the other two vectors (Table 1, Fig. 2).

We have also analyzed the effect of different concentrations of IPTG or MTG on the fold induction of luciferase activity in *lacI*-expressing HT1080 or NIH 3T3 cells, transduced with each of the three inducible vectors. As shown in Fig. 3, IPTG and MTG showed similar induction efficiency for all vectors in both cell types, with the inducer concentrations as low as 1.25 mM (for IPTG) or 1.25–5 mM (for MTG) sufficient for maximal induction.

3. Conclusions

(1) We have constructed and tested a series of retroviral vectors with internal promoters that are regulated by β-galactosides via LacI-based transactivator or repressor proteins. Expression from *lac* operator-containing enhancerless promoters was dependent on LAP activator proteins in transient transfection assays, but these promoters were fully active even in the absence of LAP in stable retrovirus-infected cell populations. This failure to regulate an enhancerless promoter most likely reflects specific integration of retroviruses in transcriptionally active regions, near cellular enhancer sites (Sandmeyer et al., 1990; Sablitzky et al., 1993). In contrast, cell populations stably infected with retroviruses containing complete promoters coupled with *lac* operator sequences were efficiently inhibited by LacI repressor and showed β-galactoside inducible expression. Very recently, Paulus et al. (1996) reported that retroviral vectors containing an enhancerless promoter dependent on a tetracycline-inhibited transactivator protein were appropriately regulated by tetracycline, when the expression was analyzed 48 h post-infection. The long-term regulation of these vectors in stably infected cell populations, however, has not been tested.

(2) We have developed three LacI repressor-regulated retroviral vectors, containing RSV or CMV-derived internal promoters in *anti* orientation relative to LTR. These vectors differ in their maximal expression levels and inducibility by β-galactosides. As compared to a very efficient constitutively expressing retroviral vector LNCX, these vectors provide 3 to 50-fold lower expression in human and mouse cells and one order of magnitude lower retroviral titer. These levels, however, should be adequate for most *in vitro* applications requiring efficient gene transfer and the ability to regulate gene expression in mass populations of independently transduced cells. In particular, these vectors should be applicable for expressing and selecting cDNAs that promote cell growth arrest or apoptosis, or genetic suppressor elements (Holzmayer et al., 1992; Roninson et al., 1995) that inhibit essential cellular genes (Pestov and Lau, 1994). The differences in the expression levels and inducibility among the vectors developed in the present study allow one to fine-tune the expression and analysis of genetic elements with different inhibitory effects.

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Inducible retroviral vectors regulated by *lac* repressor in mammalian cells

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Molecular determinants of drug response: a genetic suppressor element analysis

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Historically, most studies of cellular drug resistance have been based on selection and analysis of drug-resistant mutant cell lines. While this approach has yielded several powerful mechanisms of drug resistance, there are inherent limitations in the spectrum of changes that can be selected in mutant cells. The present review deals with a different approach, aimed at identifying the complex of genes that determine drug response in unselected tumor-derived cell lines. This approach is based on functional selection of genetic suppressor elements (GSEs), short gene fragments that interfere with the function of the gene from which they are derived. GSEs derived from genes that potentiate drug cytotoxicity (drug-sensitivity genes) increase cellular drug resistance, while GSEs from genes that protect cells from drugs (drug-resistance genes) increase cellular drug sensitivity. Using expression libraries of randomly fragmented complementary (c)DNA cloned in retroviral vectors we have isolated GSEs from several drug-resistance and drug-sensitivity genes. Single-gene GSE analysis yields specific gene inhibitors and delineates protein domains involved in functional interactions. This experimental strategy has also been used to identify novel drug-sensitivity genes, by using normalized random fragment libraries of total cellular cDNA to select GSEs and GSE combinations that confer drug resistance. Analysis of genes identified through this approach yields important information with potential clinical relevance.

Key words: Cellular drug resistance, cellular drug sensitivity, cytotoxicity, genetic suppressor elements.

Multiple determinants of cellular drug response

The killing of a tumor cell by a cytotoxic chemotherapeutic drug is a complex process, the outcome of which depends on many cellular functions [1]. The general steps of this process are illustrated schematically in Figure 1. While some drugs (e.g. doxorubicin or taxol) can enter the cell by passive diffusion through the lipid bilayer of the plasma membrane, other agents (such as methotrexate) require the presence of special carriers in the membrane for their entry. Some of the drugs have little or no activity in the form in which they are administered and need to be activated through chemical modification by appropriate cellular enzymes (e.g. polyglutamation of methotrexate or metabolic conversion of cyclophosphamide). An active drug inside the cell has to reach and bind its cellular target molecule (DNA, microtubules, etc.). This interaction eventually results in damage to the target, which should then escape repair by cellular repair enzymes. With most drugs, however, the formation of the drug-induced lesion is still insufficient to produce a cytotoxic response. Instead, lesion formation may trigger one of the cellular programs that result in cell growth arrest or programmed cell death (apoptosis). The p53 tumor suppressor gene has been identified as a major positive regulator of the pathways that lead to growth arrest or apoptosis in response to cellular damage [3–5]. Drug-induced apoptosis is also subject to regulation by a family of proteins related to the oncogene *BCL2* [5–7]; some members of the family including *BCL2* inhibit apoptosis, and others such as *BAX* promote cell death. In the past most studies on the cellular mechanisms of drug response were carried out on mutant cell lines isolated by multistep selection for high levels of drug resistance. This type of selection favors dominantly acting genes such as *MDR1* [8] or *MRP* [9], the products of which act directly on the drugs or their derivatives and that increase cellular drug resistance in proportion to their expression. The limitations of this approach are apparent from the fact that some of the most general drug resistance mechanisms, such as increased *BCL2* expression or p53 inactivation, have not yet been reported in drug-selected mutant cell lines.

From the above brief outline, it is apparent that there are many potential cellular changes that can lead to drug resistance. The active resistance mechanisms, involving

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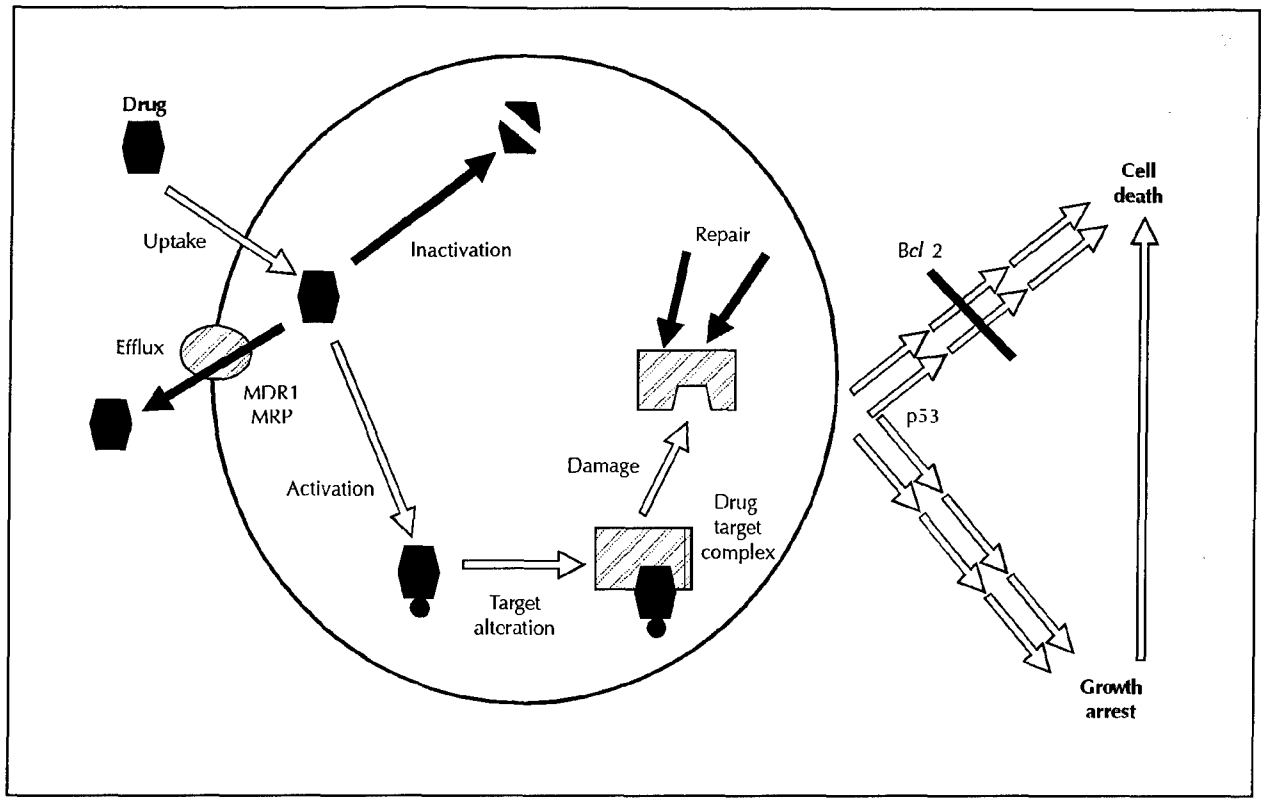

Figure 1. Determinants of drug-induced cytotoxicity. Solid arrows indicate cellular functions that interfere with the drug effect. White arrows indicate cellular functions that potentiate drug-induced cytotoxicity. MDR, multidrug resistance; MRP, multidrug resistance protein. From [2] with permission.

increased expression of genes responsible for cellular defense, are indicated with black arrows in Figure 1. These mechanisms include metabolic drug inactivation, active extrusion of the drugs and damage repair. Some of the drug resistance mechanisms are specific for individual classes of drugs, while others affect different classes of agents and induce a phenotype known as multidrug resistance (MDR). The best known MDR mechanisms are mediated by broad-specificity efflux proteins, including MDR1 P glycoprotein [8] and multidrug resistance protein (MRP) [9]. The most pleiotropic of all the documented MDR genes, however, is the apoptosis suppressor *BCL2*: introduction of *BCL2* into *BCL2*-negative cells results in resistance to apoptosis induced by a wide variety of chemotherapeutic drugs or ionizing radiation, with no apparent exceptions [5–7].

While most previous studies in the area of drug resistance have dealt with the activation of drug-resistance genes, an even greater variety of potential resistance mechanisms would result from changes in cellular processes that potentiate rather than prevent drug damage (white arrows in Figure 1). These changes include, for example, the loss of a carrier protein responsible for drug uptake, inhibition of an enzyme that metabolically activates the drug, or a deficiency in positive regulators of programmed cell death. A better-known resistance mechanism of this type involves decreased function or expression of topoisomerase II [10]. The changes in topoisomerase II lead to drug resistance due to decreased formation of cleavable complexes between DNA and topoisomerase II. Such complexes are stabilized by topoisomerase II-interactive drugs (anthracyclines, epipodophyllotoxins, amsacrine) and their accumulation triggers subsequent cytostatic and cytotoxic responses [11]. The most pleiotropic of all the resistance mechanisms in this category is associated with mutational inactivation of p53, which, when expressed in the wild-type form in neoplastic cells, triggers the onset of apoptosis after exposure to different classes of chemotherapeutic drugs or ionizing radiation [3–4]. The resistance phenotypes resulting from decreased function of drug-sensitivity genes are genetically manifested as recessive [12]. While dominant drug-resistance genes can be readily cloned or identified by their ability to confer drug resistance upon transfer into drug-sensitive cells, the drug-sensitivity genes were, until recently, refractory to gene transfer analysis.

The genetic suppressor element approach to drug response

Over the past several years our laboratory has been using a general approach for identifying the molecular determinants of drug response in tumor cells that had not been selected for drug resistance *in vitro* [2]. In this approach we ask the following question: inhibition of which genes in a tumor-derived cell line would increase or decrease cellular resistance to chemotherapeutic agents? Genes and gene products identified through this approach can then be used as targets for the development of new agents that would either augment or inhibit the function of such genes, in order to sensitize tumor cells to anticancer drugs. The key to this experimental strategy is expression selection of genetic suppressor elements (GSEs), short biologically active complementary (c)DNA fragments encoding peptides that act as dominant inhibitors of protein function or antisense RNA molecules that efficiently inhibit gene expression. GSEs in most cases act in the opposite direction to the gene from which they are derived. Thus GSEs arising from drug-sensitivity genes would confer drug resistance, while GSEs from drug-resistance genes would render cells more sensitive to the drugs. The ability to use GSEs from drug-sensitivity genes as dominant selectable markers is particularly important, as it allows one to analyze drug-sensitivity genes through the same type of gene transfer techniques that have proven so valuable in the drug resistance gene studies.

The strategy for the isolation of GSEs is illustrated in Figure 2. The first step of the procedure involves preparation of an expression library containing randomly fragmented cDNA of the gene or genes targeted for suppression. During library construction, all the cDNA fragments are supplied with translation initiation and termination codons so that they can give rise to peptides if expressed in the sense orientation. The expression library is introduced into the appropriate recipient cells, which are then selected for the desired phenotype (e.g. drug resistance). The vectors (or their inserts) are then recovered from the selected cells and used as a GSE-enriched first order library for one or more additional rounds of selection. Eventually, cDNA fragments enriched by the selection are used for functional testing as individual clones. Sequence analysis of functionally active GSEs is then used to determine whether they are sense- or antisense-oriented, what part of the gene they come from and, if the starting material for library construction included multiple genes, which genes give rise to such GSEs. Isolation of GSEs from a random fragment library prepared from a single gene or cDNA allows one to develop specific genetic inhibitors for this gene and to map out functional domains of the corresponding protein (encoded by sense-oriented GSEs). On the other hand, isolation of GSEs from a complex mixture of genes (such as total cellular cDNA) provides the means to identify previously unknown genes involved in the phenomenon under study.

Isolation of GSEs from drug-sensitivity and drug-resistance genes

The random fragment selection strategy was first shown to work in bacteria, using bacteriophage lambda as a model system [13]. In subsequent studies we have applied this method to mammalian cells, using retroviral vectors for library construction and functional analysis of individual clones. In the first project we have selected GSEs from the cloned cDNA of topoisomerase II, which potentiates the cytotoxic action of drugs that stabilize the cleavable complex formed by two molecules of topoisomerase II covalently bound to cellular DNA nicked in both strands [11]. GSEs that reduce cellular levels of topoisomerase II or interfere with cleavable complex formation decrease the number of cleavable complexes formed at a given concentration of the drug and thus, presumably, make cells more resistant. Indeed, we were able to use etoposide, a topoisomerase II-interactive drug, to isolate a series of GSEs from a random fragment library of human topoisomerase II cDNA. Both sense- and antisense-oriented GSEs were isolated and shown to confer a resistance phenotype which was specific to topoisomerase II-interactive drugs [14]. Most of the peptides encoded by the sense-oriented GSEs were clustered in a highly conserved region of topoisomerase II, associated with topoisomerase II dimerization. Expression of another GSE peptide interferes with normal nuclear localization of topoisomerase II, suggesting that the corresponding domain is involved in protein transport (C. Zelnick, A.V. Gudkov, I.B. Roninson, 1993, unpublished data). Thus, the topoisomerase II study provided genetic confirmation of the mechanism of drug resistance associated with a decreased function of topoisomerase II and indicated some previously unknown functional domains of the protein.

In addition to increasing cellular resistance to topoisomerase II-interactive drugs, cell populations carrying topoisomerase II-derived GSEs showed a decrease in their growth rate. The growth-inhibitory effect of topoisomerase II GSEs suggested to us that the most efficient GSEs from topoisomerase II may have been missed by our selection, which involved growing cells that express the GSEs constitutively. To overcome this problem (which almost certainly is not limited to topoisomerase II) we have developed a set of regulated retroviral vectors, expression from which is inducible by β-galactosides [15]. We are currently investigating the applicability of these vectors to the selection of GSEs with growth-inhibitory properties.

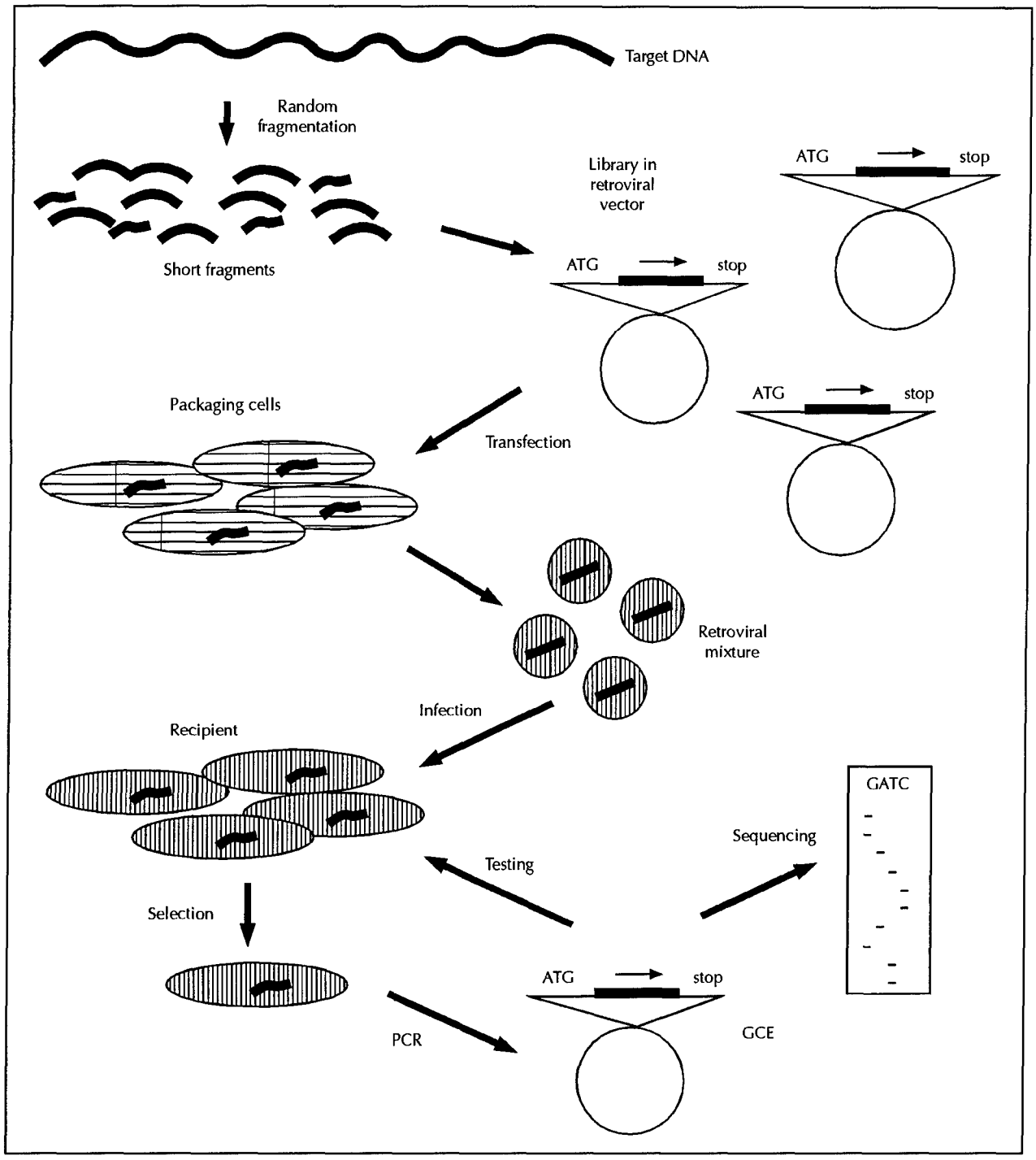

Figure 2. Selection of genetic suppressor elements in mammalian cells, using retroviral vectors. PCR, polymerase chain reaction. From [2] with permission.

The isolation of GSEs from topoisomerase II, a drug-sensitivity gene, was based on a fairly conventional drug selection procedure. In contrast, the expected property of GSEs from drug-resistance genes is to sensitize cells to the drugs, which is not usually viewed as a selectable phenotype. We have developed a strategy for the isolation of drug sensitizing GSEs using as the initial targets two pleiotropic drug-resistance genes, *MDR1* (P glycoprotein) drug efflux pump, and *BCL2* apoptosis suppressor. A common selection strategy used in both projects was physical isolation of dead or dying cells, arising after exposure to low drug concentrations that normally cause

only minimal cell death. The integrated GSEs were then recovered from the selected cells by polymerase chain reaction (PCR). A specific selection strategy, using a fluorescence-activated cell sorter (FACS), was also used for *MDR1* inhibition. This strategy is based on the ability of P glycoprotein to pump out a vital fluorescent dye rhodamine 123; *MDR1* GSEs were selected for their capacity to increase rhodamine 123 accumulation in P glycoprotein-expressing cells [16]. The *MDR1* project yielded multiple sense- and antisense-oriented GSEs; the sense-oriented GSEs encoded peptides from both nucleotide-binding domains and transmembrane regions of P glycoprotein. The *BCL2* project yielded a single sense-oriented GSE, the mechanism of action of which is still unclear.

Identifying new drug-sensitivity genes through GSE selection

Following successful isolation of resistance-inducing GSEs from topoisomerase II cDNA, the random fragment selection strategy was expanded to isolate GSEs from unknown genes, using a retroviral library containing random cDNA fragments of all the genes expressed in a mammalian cell line [17]. To enable the isolation of GSEs from genes expressed at low levels, random fragments of total cellular cDNA were subjected to normalization, a procedure which equalizes the representation of differentially expressed messenger RNA sequences in a cDNA preparation [18]. A very large library of normalized random cDNA fragments from mouse NIH 3T3 cells (3×10^7 recombinant clones) was first used to find novel genes which, like topoisomerase II, would potentiate the cytotoxicity of etoposide in NIH 3T3 cells or their derivatives [17]. No increase in etoposide resistance was observed after the first round of selection, but the sublibrary recloned from the selected cells and introduced into NIH 3T3 cells produced a noticeable increase in etoposide survival. The retroviral inserts after the second step of selection were enriched in a small number of sequences, functional analysis of which yielded three GSEs conferring etoposide resistance. Two of the GSEs showed no homology with any known genes, while the third GSE encoded antisense RNA for a heavy chain of the motor protein kinesin, which was not previously implicated in drug response. The anti-kinesin GSE induced resistance to DNA-damaging agents, and also promoted the immortalization of mouse embryo fibroblasts. Interestingly, this dual property of promoting drug resistance and immortalizing senescent fibroblasts is shared by the anti-kinesin GSEs and GSEs derived from the cDNA of p53 [19]. Downregulation of kinesin was observed not only in the GSE-transduced cells but also in several cell lines independently selected for etoposide resistance [17]. This naturally occurring mechanism of drug resistance, if identified in clinical cancer, would offer a plausible therapeutic venue, since cells with decreased expression of kinesin become hypersensitive to microtubule-depolymerizing drugs (A.V. Gudkov, I.B. Roninson, unpublished data). This example illustrates the potential clinical significance of identifying new genes involved in cellular drug response.

We also generated a normalized cDNA fragment library from human HeLa cells and used this library to select for resistance to other drugs in human tumor-derived cell lines. As in etoposide selection, we observed increased survival of cells transduced with the sublibrary and enrichment for a limited number of retroviral inserts. Most of the cloned inserts, however, when tested individually, had an intermittent effect. They produced drug resistance in some but not all assays; their effects varied for different sublines of the same recipient cell line and appeared to depend on specific tissue culture conditions. The key to this frustrating problem was found in some of the more recent selections and through analysis of the parameters of retroviral integration and gene expression in our experimental system.

Combinatorial effect of GSEs: aphidicolin selection

Inhibition of DNA replication is a common effect of many anticancer drugs, including, among others, cytarabine, methotrexate, and topoisomerase II-interactive agents. These drugs, as well as a specific DNA replication inhibitor, aphidicolin, are known to induce apoptosis [20]. To determine the extent to which the inhibition of DNA replication *per se* is responsible for drug-induced apoptosis we decided to isolate GSEs that would confer resistance to aphidicolin, an inhibitor of the elongation stage of the replication process. We transduced the HeLa-derived normalized cDNA library into human HeLa cells on a very large scale (10^8 recipient cells were infected), and carried out aphidicolin selection. Library-transduced cells showed only slightly increased survival relative to the control. A first-order library was then generated in LNCX vector from PCR-amplified proviral inserts of the selected cells. This library was introduced into HT1080 fibrosarcoma cells and produced a clear increase in aphidicolin resistance relative to vector-infected cells (Figure 3 top). A second-order library was generated from the surviving cells, and this library produced even stronger protection from aphidicolin (Figure 3 top). This strong protective effect of the second-order library has been reproduced in 10 independent infection/selection assays. Five hundred plasmid clones constituting the second-order library were characterized by single-base sequencing, to identify identical clones. Thirty-two clones were represented in multiple copies among this set, with the frequency of individual clones ranging from 0.4% to 28%. These 32 clones (in LNCX

vector) were then tested individually for their ability to confer aphidicolin resistance in HT1080 cells. Seven clones scored positive in some of the assays, but in most cases their protective effect was either very weak or undetectable, even though the second-order library invariably produced strong protection in the parallel assays.

Based on the studies described in the next section, we hypothesized that the effect of the second-order library was due to multicopy integration and cooperation between several different GSEs transduced into the same cell. In the absence of cooperating elements, individual GSEs would still be able to provide weak and heterogeneous resistance; this would explain the variable positive results with single GSEs in the functional assays and the enrichment for such GSEs after the first round of selection. To identify the cooperating elements, we isolated several cellular clones obtained after transduction with the second-order library and aphidicolin selection and recovered retroviral inserts integrated in these clones. These cellular clones were found to contain several integrated proviruses with an uneven mixture of three different sequences, derived from different genes and highly enriched in the second-order library. A mixture of these three sequences (designated 41/49) was tested for the ability to confer aphidicolin resistance, in parallel with the second order library. As shown in Figure 3, the protective effect of the 41/49 combination was as strong as that of the second-order library, a result not previously observed with any individual clones. These results indicate that aphidicolin resistance was indeed due to cooperation between different GSEs.

The finding that GSEs derived from different genes can produce drug resistance when acting in combination opens an additional field of application for the GSE strategy. It indicates that the GSE approach not only identifies individual gene products and the biochemical pathways in which they act, but also, through the cooperation of the corresponding GSEs, pinpoints interactions between the different pathways involved in drug response.

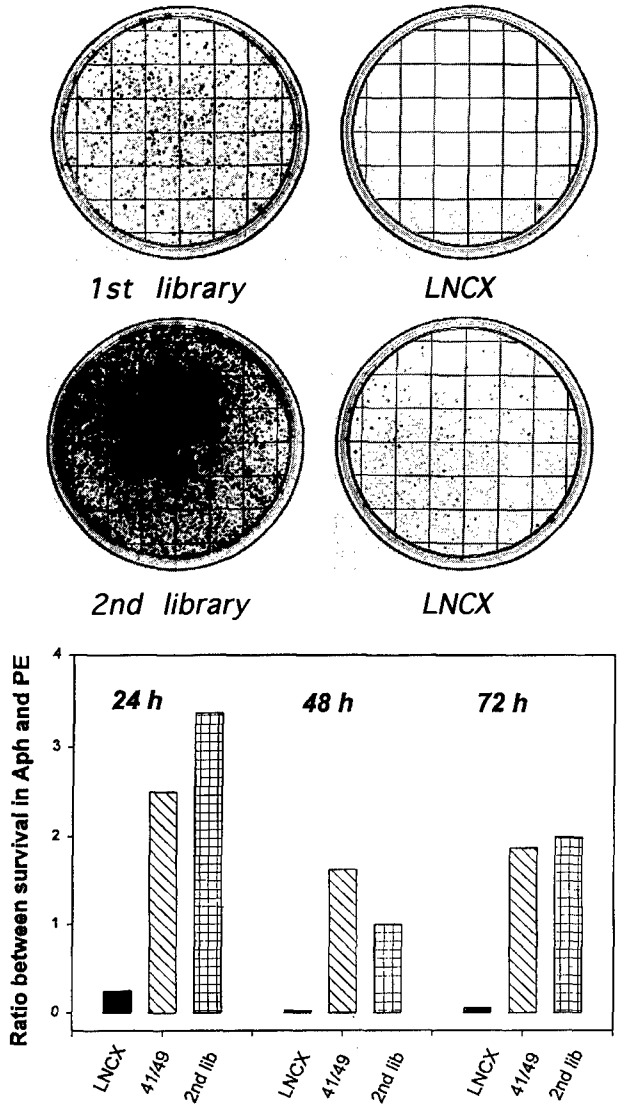

Figure 3. Isolation of a genetic suppressor element combination conferring resistance to aphidicolin in HT1080 cells (transfected with an ecotropic retroviral receptor, LNCX). *Top.* Cells infected with retroviral sublibraries isolated after the first (above) or the second (below) round of aphidicolin selection; controls were infected with insert-free LNCX vector virus. After 24 h, 10^6 cells were plated per P150 plate, and 24 h later were exposed to 4 μg/ml aphidicolin (Aph) for 72 h; surviving cells were stained with crystal violet 21 days (above) or 28 days (below) after this exposure. *Bottom.* Cells transduced with insert-free vector (LNCX), with a mixture of three clones enriched after two rounds of aphidicolin selection (41/49), or with a sublibrary derived after two rounds of selection (2nd lib). Cells were plated in six-well plates in duplicates (10^4 cells for drug treatment or 5×10^2 cells for plating efficiency control, PE). After treatment with 4 μg/ml aphidicolin for the times indicated, cells were allowed to form colonies. All plates were then stained with methylene blue and the optical density at 680 nm was determined. Cell survival is plotted as the ratio of optical density for aphidicolin-treated and PE control samples.

Methodological issues in the selection of GSE combinations

The realization that GSEs can act in combinations raises several methodological issues for GSE isolation and analysis. One of these issues is the likelihood of co-infection of the same recipient cell with several different retroviruses. Under our standard infection conditions the infection rate rarely approaches 100%, and one would normally assume that infection under such conditions would be low. We were aware, however, that this assumption was incorrect, as we had carried out a detailed analysis of retroviral in-

fection and expression in our most commonly used cell/vector system, which includes human HT1080 fibrosarcoma cells and retroviral expression vector LNCX [21] which expresses the neo (G418 resistance) gene from the MoMLV long terminal repeat promoter and the inserted gene or GSE from an internal cytomegalovirus (CMV) promoter. We have analyzed the expression of a reporter gene (luciferase) from the CMV promoter in many mass populations of HT1080 cells that were infected under the conditions resulting in different initial infection rates (IIR) and then selected with G418. This study led to the following conclusions [22]. (1) Reporter gene expression in the populations with IIR ≤ 5% is highly variable between different experiments and often very low or undetectable. The loss of reporter gene expression in such populations is due to G418-driven selection for proviruses in which the internal CMV promoter is lost or inactivated. (2) In populations with IIR ≥ 6%, reporter gene expression is positively correlated with the IIR (Figure 4a). (3) Cell populations with IIR ≥ 6% carry an average of two or more copies of unrearranged provirus per cell, with a general correlation between the IIR and the copy number (Figure 4b). Thus, multicopy provirus integration is common even under the conditions where most of the recipient cells were uninfected. (4) Among different clones and populations of infected cells, a strong positive correlation is observed between the provirus copy number and the expression of the reporter gene (Figure 4c). (5) Even in populations with higher IIR values, an increase in the duration or stringency of G418 selection can lead to the loss of reporter gene expression [22].

This analysis has demonstrated that multicopy integration of retroviral vectors is a rule rather than an exception under the standard selection and testing conditions, which

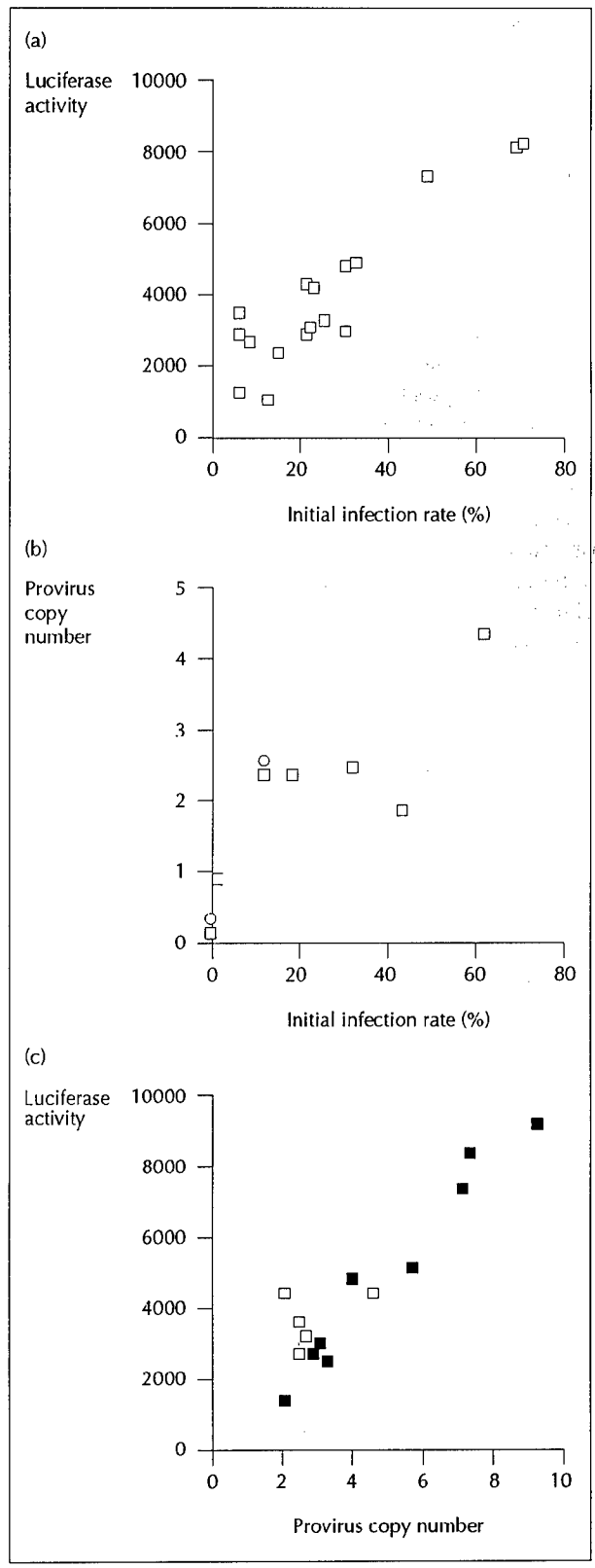

Figure 4. Relationship between initial infection rate (IIR), provirus copy number and reporter gene expression in HT1080 cells (with ecotropic receptor) infected with an ecotropic retroviral receptor (LNCX) carrying luciferase reporter gene (*LNCluc*). (a) Relationship between IIR and luciferase activity in 17 mass populations of cells independently infected with *LNCluc* at an IIR of ≥6%. Luciferase activity was measured on cells recovered in an exponential phase of growth 3–6 days after the completion of G418 selection. (b) Relationship between IIR and average unrearranged provirus copy number in *LNCluc*-infected mass populations. (c) Relationship between unrearranged provirus copy number and luciferase expression in different clones and populations of *LNCluc*-infected cells. Cell populations are indicated with open squares and clones with closed squares. From [22] with permission.

makes it possible to select active GSE combinations from retroviral libraries. On the other hand, our analysis indicated several factors that should be taken into consideration for the isolation of GSE combinations. (1) Experiments with low infection rates (≤5% in the case of HT1080 cells) are likely to fail, as co-integration of different retroviruses would be rare under such conditions, and because the GSEs may not be expressed in G418-selected populations. This concern is particularly important in the analysis of those cell types that are poorly susceptible to retroviral infection. To increase the infectability of human cell lines, we transfect them with the gene for the murine ecotropic receptor [23], which renders cells infectable with ecotropic retroviruses, and select the most infectable subclones. Furthermore, to overcome the negative effect of G418 selection, we have developed retroviral vectors that, instead of *neo*, carry the gene for the green fluorescent protein [24] as a marker that can be used for single-step non-cytotoxic selection by FACS. (2) Complete recovery of retroviral inserts is essential at intermediate rounds of selection, as a necessary component of an active GSE combination may otherwise be lost. Among different recovery methods, we have found two to be particularly reliable. The first method involves PCR amplification of cDNA inserts from integrated retroviral vectors, using vector-derived primers and *Pfu* DNA polymerase (which has a lower error rate than the most commonly used *Taq* polymerase); the recovered inserts are then recloned into the retroviral vector to generate a sublibrary of the largest possible size. The second method involves so-called long PCR amplification of the entire integrated provirus rather than just the cDNA insert. The PCR-amplified provirus is then used to transfect retrovirus-packaging cells, generating a high-titer retroviral sublibrary. In this procedure, recloning after the final round of selection is still required for sequencing and functional analysis of the enriched GSEs. (3) Candidate GSE clones should be tested both individually and in different combinations, and the sublibrary of clones recovered after the latest round of selection should be used as a positive control for functional testing.

Concluding remarks

Our results demonstrate that GSEs inhibiting known or unknown genes involved in drug response can be isolated by expression selection of random cDNA fragments that confer resistance to various drugs. GSE selections from random fragment expression libraries derived from single genes have allowed us to develop genetic inhibitors of specific drug-sensitivity and drug-resistance genes. Furthermore, selection of sense-oriented GSEs allows one to identify previously undefined functional domains of proteins that play an important role in drug response, with possible applications to rational design of drugs targeting such proteins.

Selection of resistance-inducing GSEs from normalized libraries of total cellular cDNA opens an exciting opportunity for rapid identification of numerous previously unknown drug-sensitivity genes. While characterization of the genes identified by this approach is only beginning, such characterization is likely to yield important information with potential clinical relevance (as can be seen in the example of kinesin). Furthermore, the observed cooperative effect of different GSEs makes it possible to identify active GSE combinations, the nature of which would suggest the relationship between different biochemical or regulatory pathways determining cellular drug response.

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Efficient recovery and regeneration of integrated retroviruses

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ABSTRACT

We report a rapid and efficient PCR-based rescue procedure for integrated recombinant retroviruses. Full-length proviral DNA is amplified by long-range PCR using a pair of primers derived from the long terminal repeats (LTR), and virus is regenerated by transfecting retrovirus-packaging cells with the PCR-derived provirus. The viral yield from the PCR product is similar to that from the retroviral plasmid vector, and the representation of different inserts is accurately maintained in the recovered retroviral population. This procedure is suitable for expression cloning from retroviral libraries and should be applicable to the analysis of natural retrovirus populations.

Retroviral vectors provide one of the most efficient means for gene transfer in mammalian cells. Among other applications, such vectors are used to construct high-complexity libraries for expression cloning of genes (1–3) or genetic suppressor elements (GSEs) (4–6). An integral step in expression cloning is vector recovery from the cells selected for the phenotype of interest; the recovery should be efficient and should adequately reproduce the complexity of the insert sequences present in the selected cells. The usual procedure for the recovery of integrated retroviral vectors involves PCR amplification of inserts from integrated proviruses, which is followed by a labor-intensive step of recloning the PCR products (4). The alternative biological rescue procedures (superinfection with helper virus or fusion with retrovirus-packaging cells) are apt to change the representation of different inserts in the rescued virus population due to differences in virus production by different cells. We have now developed a protocol for rapid and efficient recovery and regeneration of integrated proviruses, which does not require cloning and maintains sequence representation in the recovered virus population. This protocol uses long-range PCR (7,8) to recover functional proviral DNA, which is then used to generate retroviral particles by transient transfection of retrovirus-packaging cells.

The recovery protocol has been developed for the most commonly used type of retroviral vectors based on Moloney murine leukemia/sarcoma viruses and typified by LNCX (9). As illustrated in Figure 1A, the pLNCX plasmid vector contains different 5′ and 3′ LTR sequences; after reverse transcription, both LTR of the integrated provirus acquire the U3 region from the 3′ LTR and the U5 region from the 5′ LTR (10). To amplify the full-length proviral DNA, we have used a sense-oriented (LTRs) primer based on the U3 sequence of the 3′ LTR (5′-AATGAAAGACCCCACCTGTAGGTTTGGCAAGCTAG-3′) and an antisense-oriented (LTRas) primer from the U5 region of the 5′ LTR (5′-CAAATGAAAGACCCCCGTCGTGGGTAGTCAATCAC-3′). Genomic DNA was extracted from retrovirus-transduced cells using Qiagen Blood and Cell Culture DNA kit (high molecular weight and purity of the DNA preparation are critical for the procedure).

Each PCR reaction (50 µl) contained 0.2 mM each of the four dNTPs, 0.5 µg each of LTRs and LTRas primers and 0.5 µg genomic DNA template. In earlier experiments, PCR was carried out in Taq extender buffer (Stratagene) using 10 U *Taq* DNA polymerase (Promega) and 10 U Taq Extender (Stratagene) per tube. In more recent experiments, we have utilized instead TaqPlus Long low-salt buffer and 5 U of TaqPlus Long polymerase mixture (Stratagene); these conditions provided higher and more reproducible PCR yield. PCR was performed in a Perkin Elmer Cetus thermocycler under the following conditions: 3 min at 94°C; 27 cycles of 1 min at 94°C, 1 min at 65°C, and 2.25 min at 72°C; followed by 5 min at 72°C. Figure 1B (lane P) shows the result of a reaction carried out on the DNA from human HT1080 cells transduced with LNCX. The reaction yields two products, a short 0.7 kb band corresponding to the LTR, and a long 4.1 kb band corresponding to the full-length provirus. These conditions have been successfully used to amplify proviral DNA for LNCX or LXSN (9) based vectors (tested with inserts of up to 1.7 kb in the cloning site) in several different types of human cells. In contrast, the same PCR conditions applied to murine cells transduced with the same vectors yielded almost no full-length proviral DNA detectable by ethidium bromide staining, due to cross-reactivity of the LTRs and LTRas primers with LTR of endogenous murine retroviruses. Amplification of proviral DNA from murine cells was made possible, however, by carrying out a second round of PCR on the provirus-size DNA which was gel-purified (without ethidium bromide staining) after the first round of PCR. This is illustrated in Figure 1C, where genomic DNA template was isolated from murine NIH 3T3 cells that were infected with retroviral vector LRSN, which carries an S65T mutant form of the green fluorescent protein (GFP) (11) in the LXSN vector. After the first round of PCR, 15 µl of the reaction were used for electrophoresis in a 1% agarose gel, and DNA was extracted from the region of the gel corresponding to 3.5–4.5 kb, using QIAquick Gel Extraction Kit (Qiagen). 1/3000 of the recovered DNA was used

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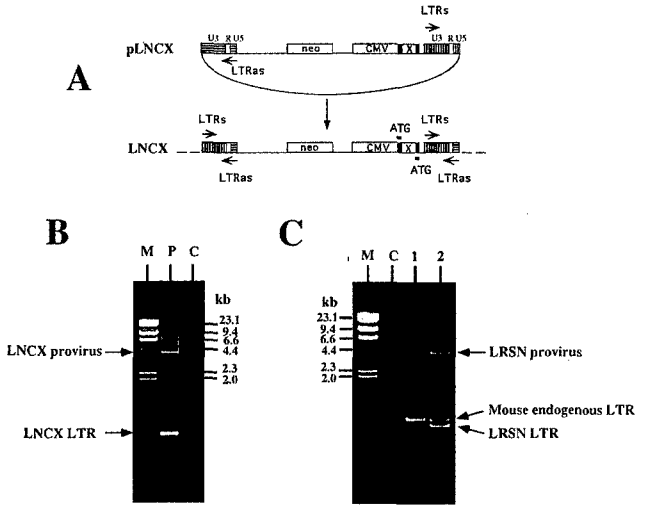

Figure 1. (**A**) Map of LNCX retroviral vector in the form of a plasmid (pLNCX) or integrated provirus. neo, neomycin phosphotransferase gene; CMV, cytomegalovirus promoter; X, insert in the cloning site of the vector. U3, R and U5 indicate the different LTR regions (10). Positions of the primers used for PCR are indicated (LTRs, LTRas, ATG). The 5′ LTR of pLNCX (horizontal stripes) is derived from Moloney murine sarcoma virus, and the 3′ LTR (vertical stripes) from Moloney murine leukemia virus; the origin of the LTR sequences in the provirus is indicated by the corresponding stripes. (**B**) Electrophoretic analysis of a long-range PCR of the DNA from HT1080 cells transduced with the LNCX vector (1% agarose gel). M, size markers (*Hin*dIII-digested λ DNA); P, PCR reaction; C, negative PCR control (no DNA template). (**C**) Electrophoretic analysis of a second round of long-range PCR (see text) of the DNA from NIH 3T3 cells that were uninfected (lane 1) or infected with the LRSN vector (lane 2). Lanes M and C are as in (B).

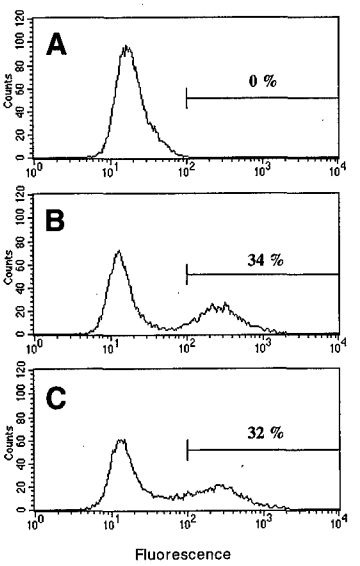

Figure 2. Flow cytometric profiles of HT1080 cells, uninfected (**A**) or infected with retrovirus produced by BOSC23 packaging cells that had been transfected with 1 μg of LRSN supercoiled plasmid vector (**B**) or 1 μg of the PCR-derived LRSN provirus (**C**). 5×10^5 cells were suspended in phosphate buffered saline containing 1 μg/ml propidium iodide (PI). Cells were analyzed with FACSort (Becton-Dickinson) using argon laser excitation (488 nm). PI fluorescence was detected in FL3 emission channel (650LP filter); GFP fluorescence of PI-negative (living) cells was monitored using FL1 emission channel (530/30 BP filter). The data were collected on log scale.

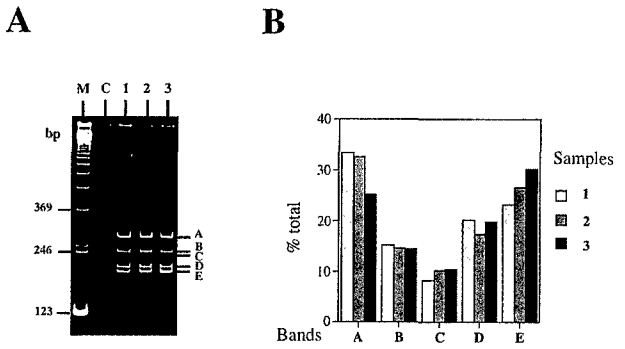

Figure 3. Short-range PCR analysis of representation of different-size inserts in a population of LNCX-derived proviruses. The following templates were used for PCR: genomic DNA of HT1080 cells carrying the integrated provirus population (sample 1), proviral DNA recovered from the same cells by long-range PCR (sample 2), genomic DNA from HT1080 cells transduced with the rescued provirus population (sample 3). Five distinct inserts are designated A–E. (**A**) Electrophoretic analysis of the PCR products in 6% polyacrylamide gel. M, size standards (123 bp ladder); C, negative PCR control (no DNA template). (**B**) Relative intensity of bands A–E in samples 1–3, as measured in ethidium bromide-stained polyacrylamide gel using ISO 1000 gel imaging system (Alpha Innotech). The intensity of each band is represented as the percentage of total intensity of all five bands for a given sample.

for the second round of PCR under the same conditions. This PCR, when carried out on the DNA from uninfected NIH 3T3 cells, yielded a single band presumably corresponding to the endogenous retrovirus LTR, traces of which remained in the gel-purified sample. In contrast, genomic DNA from LRSN-infected cells yielded bands corresponding to the endogenous and vector-derived LTR, as well as a full-length 4.1 kb LRSN provirus (lane 2).

PCR-amplified proviral DNA (combined with salmon sperm carrier DNA to a total of 15 μg) was used to transfect BOSC 23 ecotropic retrovirus-packaging cells (12); the transfection and subsequent infection of recipient cells were carried out as previously described (13). In some experiments, PCR-amplified LNCX-based provirus, recovered from HT1080 cells by a single round of PCR, was purified using QIAquick PCR Purification Kit (Qiagen) and products of one to five PCR reactions were used for transfection. Infected cells were obtained under these conditions, but at a relatively low (<3%) rate. To maximize the viral yield from the provirus derived by a single round of PCR, we gel-purified proviral DNA from a mixture of 20 PCR reactions prior to transfection (without ethidium bromide staining). The efficiency of infection with the LRSN virus recovered under these conditions and the ability of this virus to express functional GFP and Neo proteins were evaluated either by the percentage of fluorescent cells expressing GFP (as measured 3 days after infection) or by the formation of G418-resistant colonies. Figure 2 shows the fluorescence profiles of HT1080 cells (expressing the murine ecotropic receptor, 14) that were either uninfected or infected with retrovirus produced by BOSC23 cells after transfection with 1 μg of LRSN plasmid DNA or the same amount of gel-purified LRSN proviral DNA. The percentage of cells infected with the plasmid-derived virus was 34% by fluorescence and 30% by G418 resistance, while the corresponding values for the recovered virus were 32 and 46%, indicating that the PCR-generated linear provirus was transcribed in BOSC23 cells as efficiently as the supercoiled plasmid. In the case of LRSN recovered from murine

NIH 3T3 cells by two rounds of PCR, the product of a single PCR reaction (purified using QIAquick PCR Purification Kit) yielded the infection rate of 7.6% by fluorescence and 10% by G418 resistance (data not shown).

The maintenance of sequence representation in the recovered retrovirus population is illustrated by an experiment carried out on HT1080 cells that were infected with a normalized cDNA fragment library in the LNCX vector and selected for resistance to taxol (E.S.K., unpublished). The cDNA inserts from retroviral vectors integrated in this subpopulation were amplified by PCR using a primer (ATG) corresponding to the adaptor sequence flanking the inserts (5). Figure 3A shows electrophoretic analysis of PCR products amplified directly from genomic DNA, from full-length proviral DNA recovered by long-range PCR, or from genomic DNA of HT1080 cells infected with the recovered provirus and analyzed 3 days after infection. Each lane contains five distinct bands; their relative intensity is shown in Figure 3B. The representation of different bands is very similar in all three lanes, indicating that sequence representation in this relatively simple population has been maintained throughout the procedure.

In summary, the described provirus recovery protocol is rapid (3 days from genomic DNA extraction to the generation of infectious virus), effficient, and capable of maintaining sequence representation in the retroviral population. This protocol should be useful not only for expression cloning in retroviral vectors but also for functional analysis of sequence variability of full-length genomes in naturally occurring 'quasispecies' of different retroviruses (including HIV).

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DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 10 Aug 98

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-OCP, Fort Belvoir, VA 22060-6218

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